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The study of anti-viral properties of Trichosanthin on Turnip Mosaic Virus

By

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Abstract

Exogenous application of recombinant Trichosanthin (TCS) protein, a ribosome inactivating protein found in the root tuber of *Trichosanthes kirilowii* Maximowicz, inhibits the local lesion formation by turnip mosaic virus (TuMV) in leaves of *Nicotiana tabacum* and causes the delay in development of mosaic symptom by TuMV in *Brassica parachinensis*. A dose-response relationship was observed indicating the effectiveness of TCS in inhibiting the plant viral infection. To produce transgenic plants with TCS cDNA expressed, a chimeric gene which contains a fusion between the cauliflower mosaic virus 35S gene promoter, the TCS encoding cDNA and the octopine synthase 3′ polyadenylation sequence (ocs 3′) was constructed. The chimeric 5′35S-TCS-ocs3′ gene was cloned into a binary vector carrying neomycin phosphotransferase encoding gene for kanamycin resistance. The constructed vector was then introduced into the genomes of *Nicotiana tabacum* var. Wisconsin 38 via *Agrobacterium tumefaciens* by T-DNA mediated transformation. Three transgenic plants which were resistance to kanamycin and had been shown to synthesize TCS protein by

Western blotting, were highly resistant to the mechanical inoculation of TuMV. Efficient shoot regeneration from the internode-stem segment explants has been established in *B.parachinensis* with the ultimate goal to produce virus resistant transgenic plants of this local species.

Contents

Acknowledgements i

Abstract ii

Contents iv

Abbreviations x

Chapter 1 Introduction

1.1. Trichosanthin 1

1.2. Anti-plant viral and fungal properties of RIPs 3

1.3. *Agrobacterium*-mediated transformation 5

1.3.1. Ti (tumor inducing) plasmid 6

1.3.2. Role of *vir* proteins in T-DNA transfer 6

1.3.3. Integration of T-DNA into plant genome 11

1.3.4. Use of *Agrobacterium* plasmid as transformation vectors 13

1.4. Objective and strategy of producing transgenic plants that express TCS 15

Chapter 2 Materials and Methods

2.1. Bacterial Strains used 19

2.2. General Techniques 20

2.2.1. Growth of bacterial strains 20

2.2.2.	Restriction Enzyme Digestion of DNA	21
2.2.3.	Agarose Gel Electrophoresis of DNA	21
2.2.4.	Purification of DNA fragments from Agarose Gel using GeneClean II® (BIO 101 Inc.) kit	22
2.2.5.	Purification of DNA fragments by Phenol/Chloroform Extraction	23
2.2.6.	Ligation of DNA fragments	24
2.2.7.	Preparation and Transformation of *Escherichia coli.* Competent Cells	24
2.2.8.	Minipreparation of Plasmid DNA	26
2.2.9.	Preparation of Plasmid DNA using Magic™ Minipreps DNA Purification kit from Promega	27
2.2.10.	Preparation of Plasmid DNA using Qiagen-pack 100 Cartridge	29
2.2.11.	SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)	30
2.2.12.	Western Blot detection of TCS	33
2.2.13.	Polymerase Chain Reaction (PCR)	34
2.3.	**Construction of Plant Transformation Vectors**	**36**
2.3.1.	Construction of pSLJ 58210	36
2.3.2.	Construction of pSLJ TCS1 and pSLJ TCS2	38
2.3.3.	Conjugation of pSLJ TCS1 and pSLJ TCS2 into *A. tumefaciens* by Triparental Mating	41

2.4. Transformation of Tobacco Leaf Explants by *Agrobacterium tumefaciens* **43**

2.4.1. Growth of *A.tumefaciens* LBA4404 (pSLJ TCS1) 43

2.4.2. Surface Sterilization of tobacco leaves 43

2.4.3. Inoculation of tobacco leaf explants with *A.tumefaciens* LBA4404 (pSLJ TCS1) 44

2.4.4. Regeneration of shoots from Transformed explants 45

2.4.5. Rooting of Transformed shoots 45

2.4.6. Re-establishment of cultured Plantlets in soil 45

2.5. Analysis of the Regenerated Transgenic Tobacco **46**

2.5.1. Isolation of plant leaf protein 46

2.5.2. SDS-PAGE and Western blot detection of TCS 48

2.5.3. Anti-viral assay of Transgenic tobacco against TuMV 48

2.6. Bioassay of Inhibitory activity of TCS protein against TuMV **49**

2.6.1. Preparation of biologically active TCS protein 49

2.6.2. Purification of TuMV from infected plant leaves 51

2.6.3. Mechanical Inoculation of virus onto host plant 53

2.6.4. Anti-viral assay on Local Lesion host 54

2.6.5. Anti-viral assay on Systemic host 55

2.7 Establishment of the plant culture medium for efficient Regeneration from tissue explants of *Brassica parachinensis* **56**

2.7.1. Preparation and Sterilization of culture medium 57

2.7.2. Preparation of Sterile seedlings of *B.parachinensis* 57

2.7.3. Regeneration from Cotyledon petiole and Hypocotyl segment explants 58

2.7.4. Regeneration from Internode stem segment explants of shoot culture 60

2.8. Reagents and Buffers **61**

2.8.1. Media for Bacterial culture 61

2.8.2. Media for Plant tissue culture 64

2.8.3. Restriction Enzymes 66

2.8.4. Buffers for Agarose Gel Electrophoresis 66

2.8.5. DNA ligation Buffer 67

2.8.6. Reagents for preparation of *E.coli* competent cells 67

2.8.7. Reagents for preparation of Plasmid DNA 68

2.8.8. Reagents for Qiagen-pack 100 Cartridge 69

2.8.9. Reagents for SDS-PAGE 70

2.8.10. Reagents for Western Blotting 71

Chapter 3 Construction of Plant Transformation Vectors

3.1. Introduction **73**

3.2. Results **74**

3.2.1. Construction of pSLJ 58210 74

3.2.2. Construction of the recombinant binary vectors pSLJ TCS1 and pSLJ TCS 78

3.2.3. Conjugation of pSLJ TCS 1 and pSLJ TCS 2 into *Agrobacterium tumefaciens* via Triparental Mating 82

3.3. Discussion **90**

Chapter 4 Transformation of Tobacco Leaf Explants by *Agrobacterium tumefaciens*

4.1. Introduction **94**

4.2. Results **95**

4.2.1. Regeneration of leaf explants after transformation 95

4.2.2. The level of expression of TCS in transgenic tobacco leaf 100

4.3. Discussion **104**

4.3.1. Regeneration of transgenic tobacco plants 104

4.3.2. Expression of TCS in transgenic tobacco plants 108

Chapter 5 Two approaches to study the Inhibitory effect of TCS on TuMV

5.1. Introduction **112**

5.2. Results **113**

5.2.1. Expression and purification of recombinant TCS 113

5.2.2. Purification of TuMV 119

5.2.3. Anti-viral assay on local lesion host 119

5.2.4. Anti-viral assay on Systemic host 124

5.2.5. Anti-viral assay of Transgenic tobacco against TuMV 126

5.3. Discussion **129**

Chapter 6 Establishment of plant culture conditions for efficient shoot regeneration from tissue explants of *B.parachinensis*

6.1. Introduction **133**

6.2. Results **133**

6.3. Discussion **137**

Chapter 7 Conclusion

Appendix

A.1. Size of molecular weight markers **143**

A.2. References **145**

Abbreviations

APS	ammonium persulfate
BA	benzylaminopurine
bp	base pair(s)
BSA	bovine serum albumin
CaMV	cauliflower mosaic virus
DIECA	diethyldithiocarbamate
DNA	deoxyribonucleic acid
cDNA	complementary DNA
dATP	deoxyadenosine 5′–triphosphate
dCTP	deoxycytosine 5′–triphosphate
dGTP	deoxyguanosine 5′–triphosphate
dTTP	deoxythymidine 5′–triphosphate
EDTA	ethylenediaminetetraacetic acid
GTP	guanosine 5′–triphosphate
HEPES	*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid
HIV	human immunodeficiency virus
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase(s)
MAP	mirabilis antiviral protein
Mj-AMP	*Mirabilis jalapa* antimicrobial protein
MS	Murashige and Skoog
NAA	naphthaleneacetic acid

NPT	neomycin phosphotransferase
ocs	octopine synthase
PAGE	polyacrylamide gel electrophoresis
PAP	pokeweed antiviral protein
PMSF	phenylmethylsulphonylfluoride
RIP	ribosome inactivating protein
RNA	ribonucleic acid
rRNA	ribosomal RNA
RNase	ribonuclase
SDS	sodium dodecyl sulfate
TCS	trichosanthin
T-DNA	transfer-DNA
TEMED	*NNNN'* tetramethyl ethylene diamine
THF	tian hua fen
Ti-plasmid	tumor inducing plasmid of *A. tumefaciens*
TMV	tobacco mosaic virus
TuMV	turnip mosaic virus
vir	Ti-plasmid virulence region

Chapter 1
Introduction

Chapter 1 Introduction

1.1. Trichosanthin

Trichosanthin (TCS) is an active component of Tian hua fen (THF), the root tuber of the Chinese medicinal herb *Trichosanthes kirilowii* Maximowicz of the Cucurbitaceae family. THF was used for centuries in China as an abortifacient drug to terminate pregnancies (Wang *et al.*, 1986). In the early 70s, TCS was isolated and has been used for the treatment of ectopic pregnancies, hydatidiform moles and trophoblastic moles (Jin, 1985; Huang, 1987) Recently, it has been discovered that TCS can act selectively against HIV-infected cells by inactivating the HIV-replication in the infected T cells and macrophage while showing little effects in uninfected cells *in vitro* (McGrath *et al.*, 1989). Clinical studies on 51 HIV-infected patients showed that the CD4+ lymphocyte number increases while the serum HIV p24 antigen level decreases in some patients (Kahn *et al.*, 1990). The anti-HIV properties of TCS has aroused considerable research interest. The TCS cDNA has been cloned (Shaw *et al.*, 1991) and successfully expressed at high level in *Escherichia coli.* A scheme has also been developed to

purify the recombinant protein from the expressed *E.coli* (Zhu *et al.*, 1992).

TCS has been found to be a member of the type I Ribosome Inactivating Protein (RIP). Like other similar proteins such as Pokeweed Antiviral Protein (PAP), ricin and Mirabilis Antiviral Protein (MAP) from plants, TCS acts as RNA N-glycosidases by hydrolyzing a single N-glycosidic bond between adenine and ribose at A_{4324} in rat 28S rRNA (Zhang and Liu, 1992). This interrupts translation at the step of GTP-dependent binding of the elongation factor 2 (EF2) to ribosomes. Therefore, TCS is a potent inhibitor of protein synthesis.

Apart from the RIPs activity on animal ribosomes, RIPs such as PAP has been found to attack the large (25S) rRNA of tobacco ribosomes via its RNA N-glycosidase activity. Some RIPs isolated from *Dianthus barbatus* L. and *Spinacia oleracea* L. also attack ribosomes of their own plant species to generate a RNA fragment (Prestle *et al.*, 1992). This fragment contains a highly conserved sequence that is present in all 23/28 rRNAs which is considered as the putative recognition site for RIPs

(Prestle *et al.*, 1992). This suggests that RIPs inactivate plant ribosomes in the same way as that for rat liver ribosomes.

1.2. Anti-plant viral and fungal properties of RIPs

Several purified RIPs such as PAP, Abrin, Ricin, Modeccin, Gelonin and Momordin have been found to have inhibitory effects on Tobacco Mosaic Virus (TMV) local lesion production in plants by exogenous application (Stevens *et al.*, 1981). Table 1.1 summarizes the results of the experiments reported by Stevens in which the virus was mixed 1:1 with the test RIPs or with water as controls and the resulting inoculum was mechanically inoculated onto leaves of the local lesion host *Nicotiana glutinosa.*

Table 1. Effects of RIPs on local lesions production by TMV

Experiment No.	RIPs	Mean number of lesions		Inhibition (%)
		Control	Treated	
1	PAP	36	0	100
	Abrin	36	10.8	69
2	Ricin	57.6	8.3	85
	Modeccin	57.6	24.4	58
	Gelonin	57.6	28.1	51
3	Momordin	48	27.6	42

These results show that RIPs which inhibit protein synthesis also have inhibitory effects on the replication of plant virus in host plants.

Apart from the inhibition on virus, RIPs purified from barley can inhibit the growth of fungi (*Trichoderma reesei* and *Fusarium sporotrichioides*) assayed *in vitro*, and this inhibition is synergistically enhanced in the presence of enzymes chitinase known to degrade fungal cell wall polysaccharides (Leah *et al.*, 1991).

Due to these anti-viral and anti-fungal properties of RIPs, scientists undertake to produce transgenic plants that would express the RIPs and to find out whether the plants carrying these genes would be resistant to the challenge of pathogenic fungi and viruses. In recent years, transgenic tobacco plants that express a barley RIP under control of the wound-inducible promoter of the potato *wun1* gene have been produced. Transgenic plants carrying this gene exhibit increased protection against inoculation with the agronomically deleterious fungus *Rhizoctonia solani* (Logemann *et al.*, 1992).

Transgenic tobacco and potato plants expressing PAP, under control of the 35S RNA promoter from CaMV, have also been produced recently (Lodge *et al.*, 1993). It is noted that the expression of PAP in transgenic

plants exhibits resistance to infection by different groups of viruses including Potexvirus, Potyvirus and Luteovirus. Previous methods for engineering virus resistant plants include the expression of the coat protein gene (Beachy *et al.*, 1990), the replicase gene (Sccholthof *et al.*, 1993) or using the antisense RNA (Bejarano and Lichtenstein, 1992) in transgenic tobacco plants can only protect the plants against the virus or its close relatives from which the genes are derived. Since TCS shares the same RIP activity with PAP and shows amino acid sequence homology in the proposed active site (Stirpe *et al.*, 1992), this offers the possibility of developing transgenic plants expressing TCS which are resistance to plant viral infection.

1.3. *Agrobacterium*-mediated transformation

In order to produce transgenic plants expressing TCS, a plant transformation system that based on Ti-plasmid of *Agrobacterium tumefaciens* is used. *Agrobacterium*-mediated transformation is the most common system used to produce transgenic plants. In the following session, the mechanism of the *Agrobacterium*-mediated transformation (reviewed by Zambryski, 1992) will be described.

1.3.1. Ti (tumor inducing) plasmid

A.tumefaciens is a soil bacterium which can induce tumor on plants. This bacterium contains a large Ti plasmid (~200-kb) which consists of two important genetic components: transfer DNA (T-DNA) and virulence (*vir*) region (Fig. 1.1). The T-DNA, flanked by *cis*-essential 25-bp direct repeats at the left border (LB) and the right border (RB), is copied and transferred to the plant cells. The wild-type T-DNA in native Ti plasmids encodes for the synthesis of the plant growth hormones auxin and cytokinin at elevated level, resulting in an abnormal proliferation of plant cells to form gall. The *vir* region (~40-kb) consists of seven genes the *vir*A, B, C, D, E, G and H. These genes express *trans*-acting proteins for the T-DNA transfer.

1.3.2. Role of *vir* proteins in T-DNA transfer

It is known that when the susceptible plant cells are wounded, phenolic compounds such as astrosyringone are produced from the wounded plant tissue. When *Agrobacterium* is exposed to astrosyringone, the *vir*A

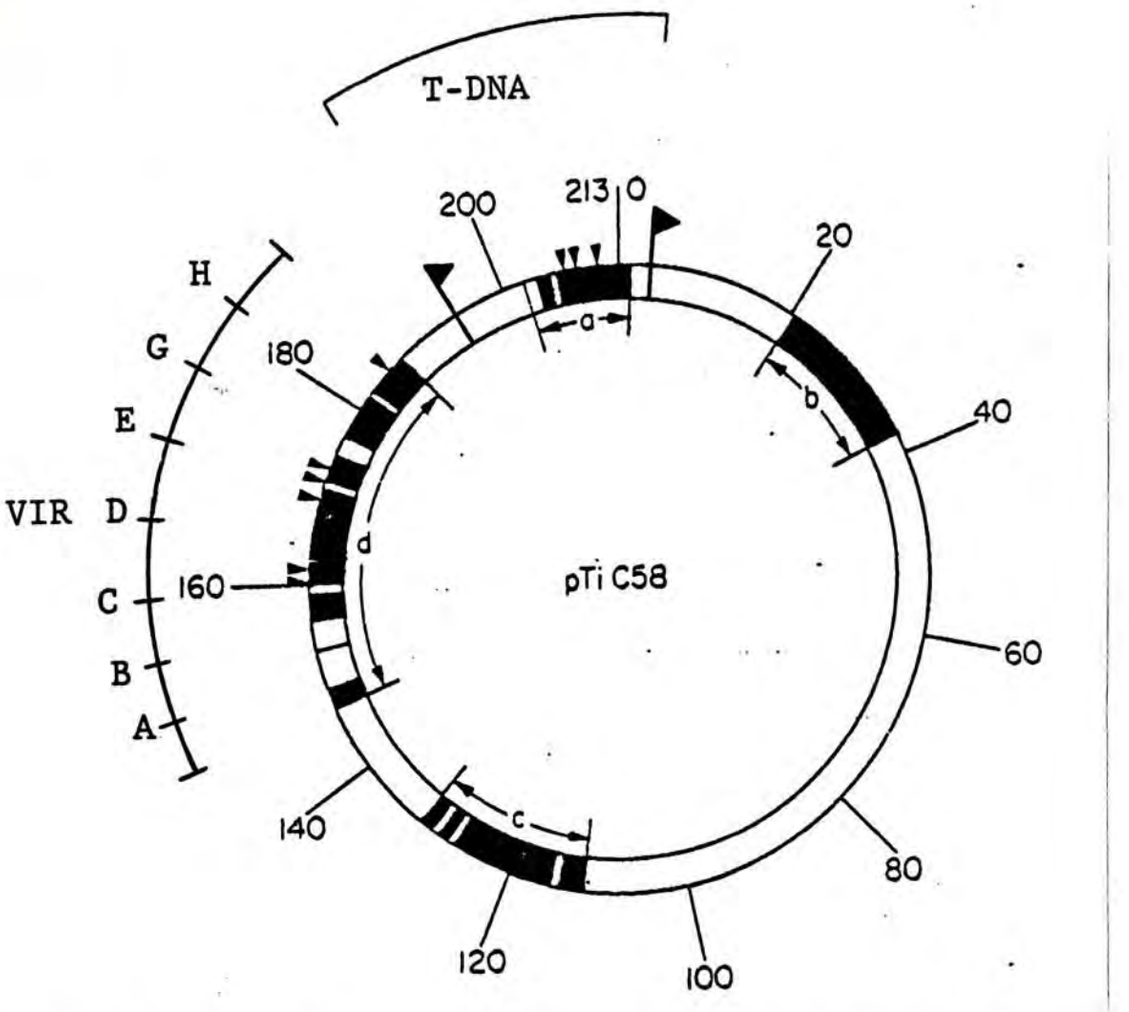


Fig. 1.1 A schematic diagram of a nopaline type Ti plasmid. Regions common to both nopaline and octopine type Ti plasmids are shown as black arcs (a-d) (Adopted from Depicker *et al.*, 1980).

protein (possibly membrane associated) recognizes and transmits this signal to activate the *vir*G protein probably by protein phosphorylation (Fig. 1.2). The altered *vir*G protein activates the rest of the *vir* genes as well as the *vir*G gene. Following the *vir* gene expression, the 25-bp repeats are possessed by a complex of *vir*D1 topiosomerase and *vir*D2 endonuclease to produce single-strand nicks at the LB and RB of the lower strand T-DNA aided by *vir*C protein. Then, the lower strand T-DNA is released by strand-displacement DNA synthesis starting from the RB and terminates at the LB. The free T-DNA directed by *vir*D2 protein at 5' end is probably bound by *vir*E2 protein forming a T-complex to protect the T-DNA against nucleases and facilitate the transport through the bacteria and plant cell membrane. Figure 1.3 shows a hypothetical *vir*B channel on the cell membrane of *Agrobacterium* which plays a role in T-DNA export.

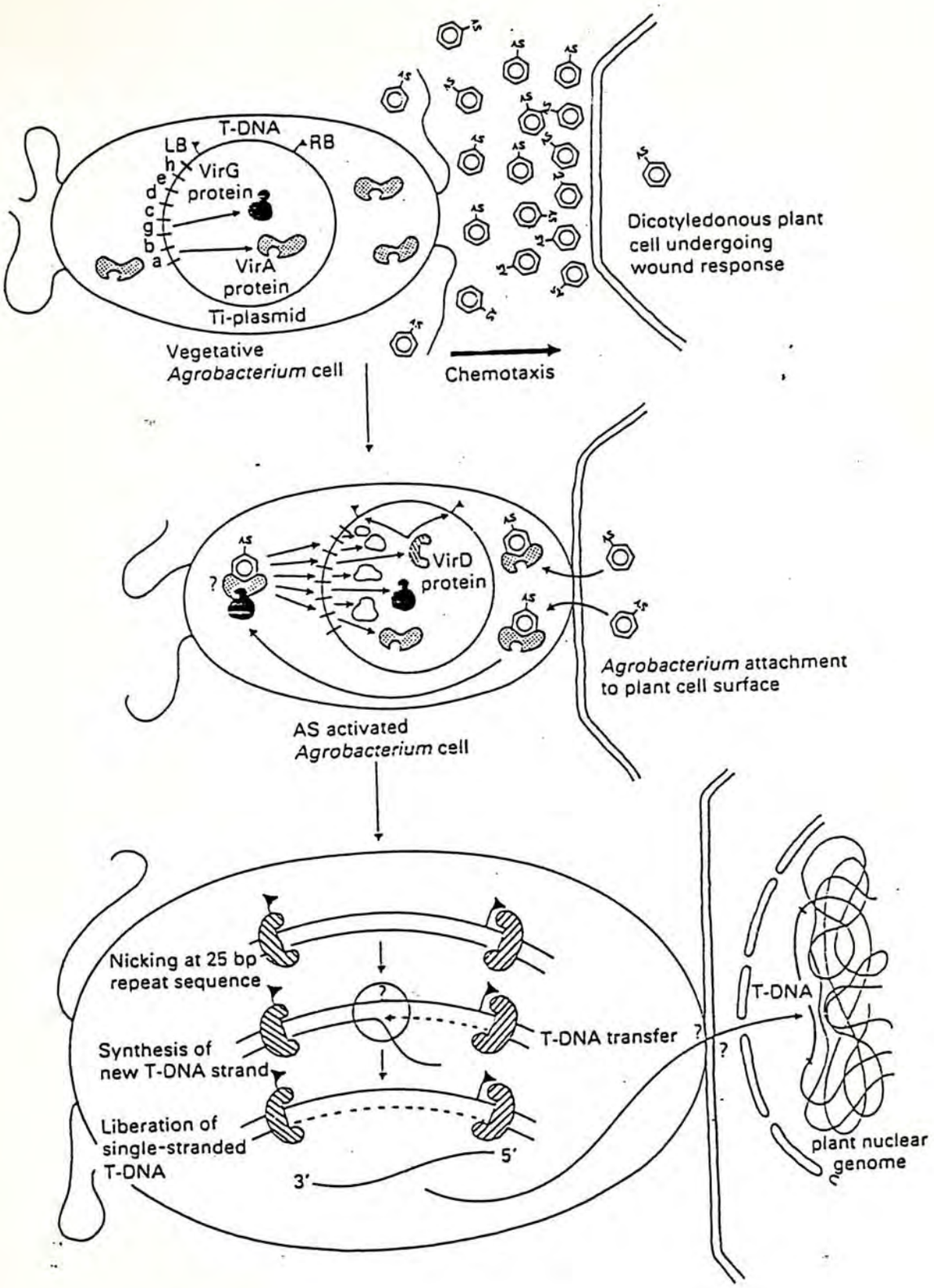


Fig. 1.2 Agrobacterium/plant interaction and mechanism of T-DNA transfer (AS = acetosyringone)

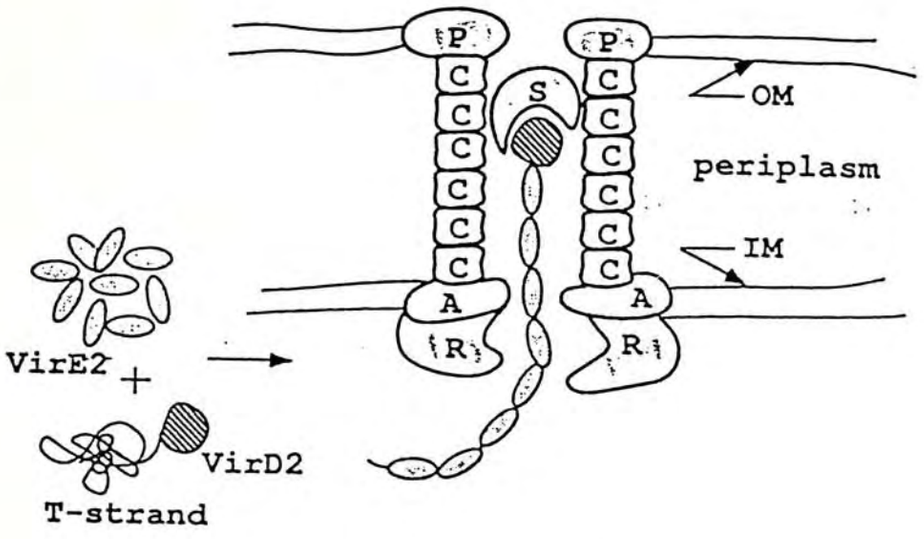
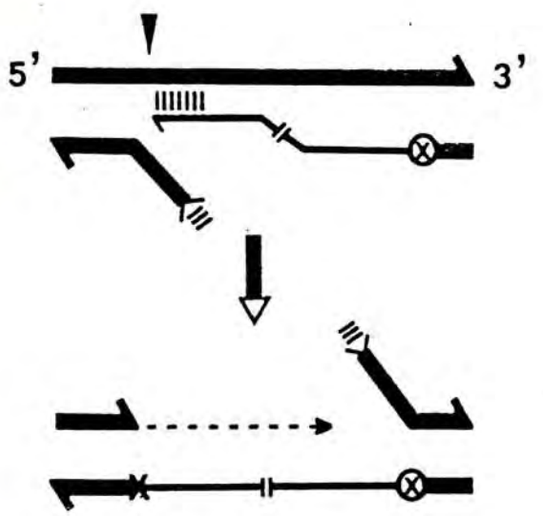


Fig. 1.3. Hypothetical *vir*B channel for T-complex export. OM, outer membrane; IM, inner membrane; P, plant-cell binding protein; S, T-complex shuttle protein; C, channel proper; A, ATPase; R, T-complex receptor (Adopted from Zambryski *et al.*, 1992)

1.3.3. Integration of T-DNA into plant genome

The T-complex is directed by the *vir*D2 protein which possesses the nuclear localizing signal sequence to the nuclear pore in plant cells. To explain the integration of the T-DNA into plant genome, a Single-Strand Gap-Repair model has been proposed (Mayerhofer *et al.*, 1991). In Fig. 1.4, the *vir*D2 protein recognizes or even causes nicks in the plant DNA and mediate the ligation of the 5′ end of T-DNA to the plant DNA. The 3′ end of T-DNA moves along the plant DNA, while the unwound plant DNA is removed by exonuclease digestion. At the position where the 3′ end of the T-DNA is stabilized by base pairing, the T-DNA is ligated to the plant DNA. At the same position a nick is introduced in the second strand of the plant DNA that initiates the plant DNA deletion in a 5′ to 3′ direction. Repair synthesis using the T-DNA as template would complete the integration process.



▼ : nick, ⫼⟩ : 5'-3' exonuclease, X : ligation, ⊗ : VirD2

----→ : repair, ⫼ : base-pairing, ▬ : plant DNA, ⊣⊢ : T-DNA

Fig. 1.4 A Single-Strand Gap-Repair (SSGR) model for T-DNA integration. This model involves VirD2-mediated recognition of a nick that is followed by ligation of the 5′ end of T-strand to the target DNA. The 3′ end of the T-strand moves along the target, while the unwound target DNA is removed by exonucleolytic digestion. The 3′ end of the T-strand is stabilized by base-pairing and ligated to the target. At the same position a nick is introduced in the second strand of the target that initiates the deletion of the target DNA and the replication of the T-strand (Adopted from Mayerhofer *et al.*, 1991).

1.3.4. Use of *Agrobacterium* plasmid as transformation vectors

It has been found that the T-DNA sequences internal to the 25-bp repeats in the Ti plasmids have no effect on the efficiency of T-DNA transfer (Binns, 1990). Thus, non-oncogenic derivatives of the native Ti plasmids, where most of the internal sequences of the T-DNA including the oncogenes have been replaced, are widely used as vectors for genetic transformation of plant cells.

We have used the *trans* vectors which are commonly called binary vectors and in our study to construct the plant transformation vectors. These binary vectors (Fig. 1.5) contain the T-DNA border sequences which link the multiple cloning sites and the antibiotic resistance gene (neomycin phosphotransferase), replicated by a broad host-range replicon. Therefore, cloning of TCS into the binary vectors can be easily manipulated using *Escherichia coli* as a host. Transfer of the engineered binary vectors into *A.tumefaciens* strain LBA4404, harbouring the Ti-plasmid with the *trans* acting *vir* region but without T-DNA and

border sequences, allows introduction of the manipulated T-DNA into the plant cells.

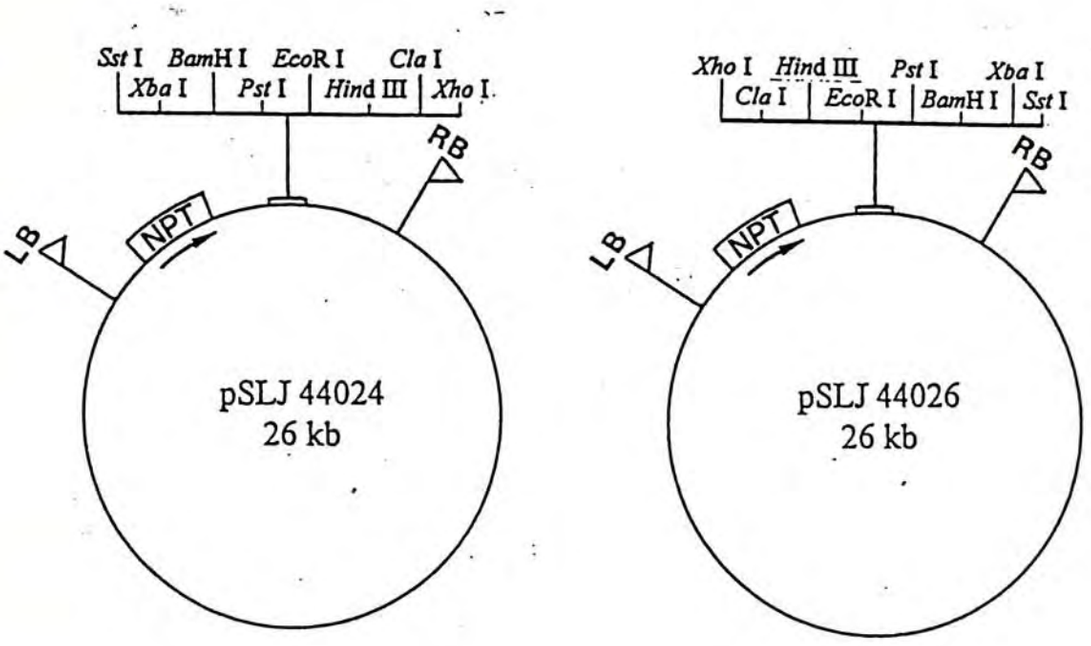


Fig. 1.5. Schematic diagram of binary vectors pSLJ 44024 and pSLJ 44026 (Adopted from Jones *et al.*, 1992).

1.4. Objective and strategy of producing transgenic plants that express TCS

In the present study, we have shown that exogenous application of TCS protects *Nicotiana tabacum* and *Brassica parachinensis* from the infection caused by turnip mosaic virus (TuMV). With these data on hand, we proceed to construct the plant transformation vectors in which the TCS encoding cDNA is under the control of the 35S RNA promoter from CaMV and is linked with the 3′ polyadenylation sequence of the octopine synthase gene (ocs 3′). This chimeric construct 5′P35S-TCS-ocs3′ is then subcloned into the DNA sequence adjacent to the antibiotic resistant gene neomycin phosphotransferase (NPT) within the T-DNA borders (RB & LB). The engineered binary vectors are introduced into *A.tumefaciens* LBA4404 via tri-parental mating and then the *Agrobacterium* is used to transform the tobacco plant cells. In the transformation, the T-DNA containing the TCS and NPT encoding sequences is transferred and integrated into the plant genome. The transformed plant cells are cultured on the selective culture medium to regenerate shoots and develop into transgenic tobacco plants habouring

the TCS encoding sequence in the genome. The transgenic tobacco plants are analyzed for the expression of TCS and for the resistance to the mechanical inoculation of TuMV. Scheme of this work is shown in Fig. 1.6.

TuMV belongs to the potyvirus group and is one of the most important plant virus in Hong Kong affecting a variety of *Brassica* species and hence causing serious loss of economically important crop plants. The virus is a filamentous particle in which a single stranded RNA is bound by 2000 molecules of capsid protein (Tremblay *et al.*, 1990) as shown in Fig. 1.7. The virus is sap-transmissible by over 40 species of aphides through the stylets. At present, there is no direct method to cure the disease except eradication of the diseased plants or spraying insecticides to kill the aphid vectors.

The study of virus resistance of transgenic tobacco expressing TCS provides us a model system to evaluate the anti-plant viral properties of TCS. The ultimate goal is to develop transgenic plants of local

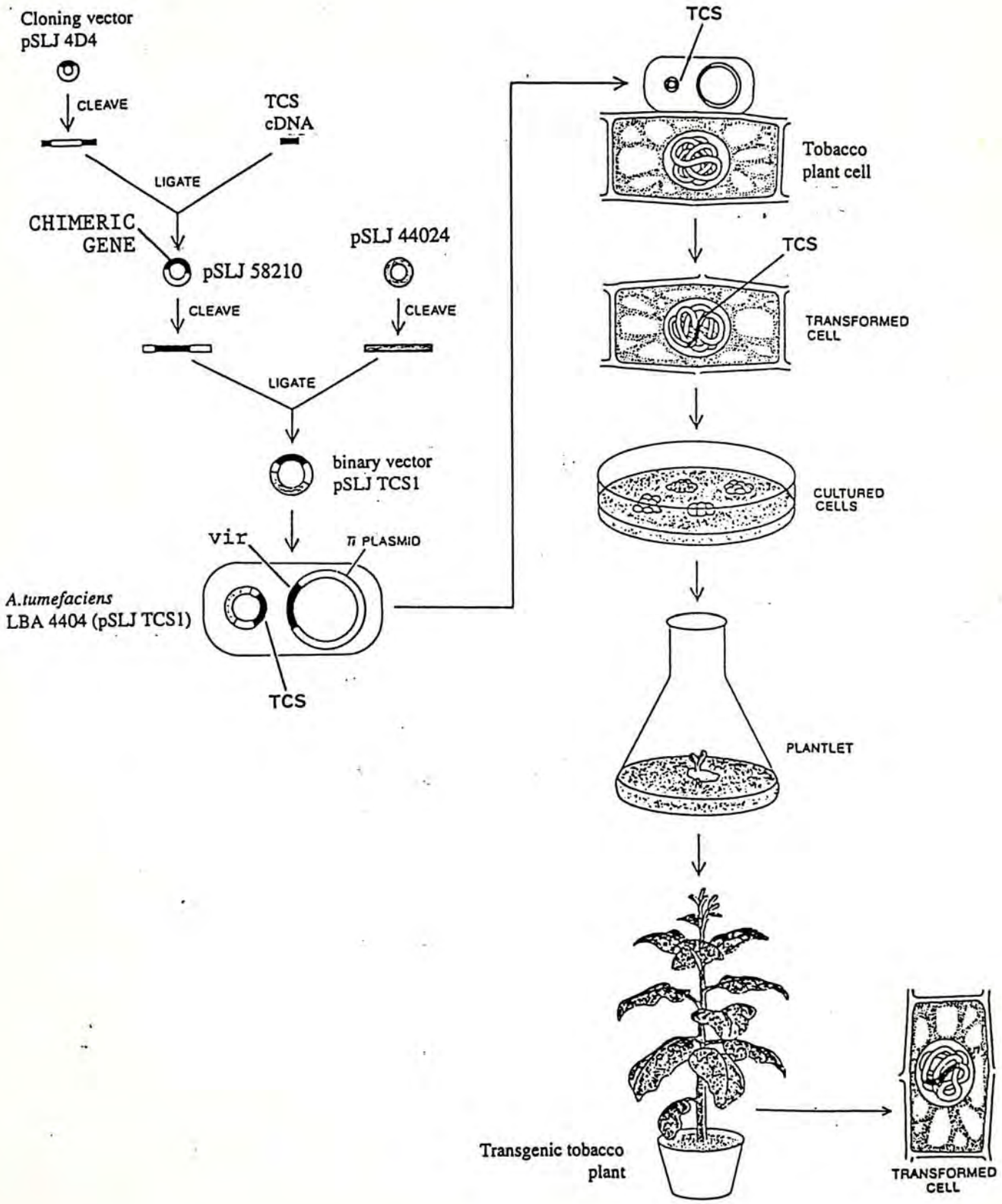


Fig. 1.6. A schematic diagram showing the strategy of generating transgenic tobacco plants.

Brassica species expressing TCS for resistant to TuMV infection. To achieve this goal, we also undertake to design an optimum nutrient conditions for the efficient regeneration of the tissue explants of *B.parachinensis* on plant culture medium, which is essential for the *Agrobacterium*-mediated transformation to produce the transgenic plant species.

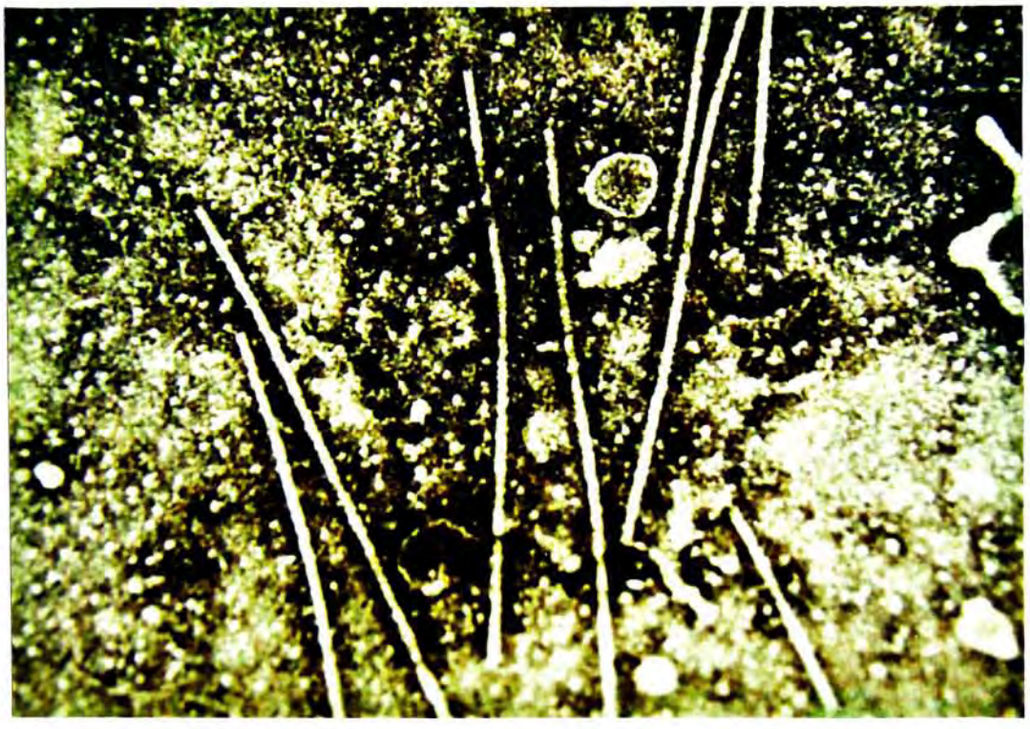


Fig. 1.7 Electron micrograph of the filamentous particle of TuMV (120,000×) (with courtesy of Ms. Wendy Ko of the Hong Kong Agricultural and Fisheries Department).

Chapter 2
Materials and methods

Chapter 2 Materials and Methods

2.1. Bacterial Strains used

Strain	Genotype	Source
E.coli DH5α	F^-, ϕ80dlacZΔM15, recA1, endA1, gyrA96, thi-1, hsdR17(r_k^-,m_k^+), supE44, relA1, deoR, Δ(lacZYA-argF)U169	Our laboratory
E.coli BL21 (DE3)	F^- ompT $hsdS_B$ (r_B^- m_B^-) with a prophage carrying the T7 RNA polymerase gene	W.F. Studier
E.coli HB101 (pRK 2013)	F^-, r_B^-, m_B^-, RecA, ara, proA, lacY, galK, str, xyl5, mtl, SupE, Km^r, ColE1 derivative with tra genes of RK2	J.D.G. Jones
A.tumefaciens LBA4404	with pAL 4404 which is a pTiAch5 (octopine plasmid) derivative with deletion of T-DNA region	J.D.G. Jones

2.2. General Techniques

2.2.1. Growth of bacterial strains

Cells were removed from the -70°C frozen stock and streaked for single colonies onto corresponding solid medium and incubated under appropriate temperature and antibiotic selection as follows:

Bacterial strains	Culture medium (Section 2.8.1.)	Temp.(°C)
E.coli BL21 (DE3, pLysS pET 58210)	M9ZB + 50 μg Ap/ml + 25 μg Cm/ml	37
E.coli DH5α	ϕb	37
E.coli DH5α (pSLJ 58210)	LB + 50 μg Ap/ml	37
E.coli DH5α (pSLJ TCS1) *E.coli* DH5α (pSLJ TCS2)	LB + 12.5 μg Tc/ml	37
E.coli HB101 (pRK 2013)	LB + 25 μg Km/ml	37
A.tumefaciens LBA4404	YEP + 0.2% glucose	28
A.tumefaciens LBA4404 (pSLJ TCS1) *A.tumefaciens* LBA4404 (pSLJ TCS2)	Min T + 500 μg Sm/ml + 50 μg Km/ml	28

Liquid culture of bacterial strains was generally started by inoculating single colonies picked from the solid medium into 5 ml of the corresponding liquid medium containing suitable antibiotics. The culture was incubated under its favourable temperature on a rotatory shaker (200 rev./min).

2.2.2. Restriction Enzyme Digestion of DNA

Restriction enzyme digestion was performed in a reaction volume of 10–60 µl. The reaction mixture containing the DNA, suitable buffer and restriction enzyme(s) was incubated at 37°C for 60–90 minutes. After digestion, the reaction products were analyzed by agarose gel electrophoresis in TBE buffer (Section 2.2.3).

2.2.3. Agarose Gel Electrophoresis of DNA

Agarose gel (30–50ml) was prepared by dissolving 0.8–2.0% (w/v) agarose (BioRad) in 1× TAE or 1× TBE buffer (Section 2.8.4) in a microwave oven. 0.5 µg ethidium bromide/ml was then added to the gel solution which was then allowed to set for at least 15 minutes. The volume of DNA samples was adjusted to 15–60 µl with water and 6× gel-loading buffer (Section 2.8.4). After loading the samples into the wells, electrophoresis was conducted at constant voltage of 50–100V in a gel tank containing 1× TAE or 1× TBE. After electrophoresis, resolved DNA was visualized on a UV transilluminator and, if necessary,

photographed by a Polaroid MP-4 instant camera installed with a HOYA R(25A) red fitter and Polaroid 667 instant film.

2.2.4. Purification of DNA fragment from Agarose Gel using GeneClean II® (BIO 101 Inc.) kit

Under ultra-violet illumination of the 1% agarose gel in TAE buffer, the band representing the desired DNA fragment was excised from the gel using a scapel blade. The weight of the gel slice was determined and 3 volumes (v/w) of NaI Solution was added and incubated at 55°C until all the agarose was dissolved. 10 µl Glassmilk® (Silica matrix) was added, inverted to mix and the mixture was kept on ice for 5 minutes. the tube was inverted occasionally to allow the Glassmilk suspended and to bind DNA efficiently. Then, the Glassmilk/DNA complex was spun down at 13,000 rpm for 30 seconds in a microcentrifuge. The pellet containing the DNA was resuspended in 500 µl NEW WASH by pipetting back and forth while digging into the pellet with the pipet tip. After it was resuspended, the complex was spun down again for 5 seconds in the microcentrifuge and the supernatant was discarded. The wash was repeated two more times.

After the last wash, 10 µl H_2O preheated at 55°C was added to elute the DNA with incubation at 55°C for 3 minutes. The eluted DNA was recovered by centrifugation at 13,000 rpm for 30 seconds and the supernatant containing the DNA was collected. The pellet was eluted for a second time and the resulting DNA portions were pooled. The yield of the DNA fragment was estimated by agarose gel electrophoresis in TBE buffer (Section 2.2.3.).

2.2.5. Purification of DNA fragment by Phenol/Chloroform Extraction

After restriction enzyme digestion, the digestion mixture was mixed with equal volume of Phenol/Choroform mixture aided with vortex. After centrifugation at 13,000 rpm for 2 minutes in a microcentrifuge, the aqueous phase containing the DNA fragment was saved. 2 volumes of absolute Ethanol was added to precipitate the DNA fragments at 4°C for 1 hour. The DNA fragment was recovered by centrifugation at 13,000 rpm for 20 minutes at 4°C in a microcentrifuge. The pellet was washed with 1 ml 70% Ethanol. After re-centrifugation for another 15 minutes, the pellet was saved and dried in oven for 30 minutes. The DNA

fragment was dissolved in 20 µl H_2O and analyzed by agarose gel electrophoresis in TBE buffer (Section 2.2.3).

2.2.6. Ligation of DNA fragments

Ligation of DNA fragments was generally performed in 10 µl reaction mixture containing DNA fragments (insert and vector), 1 µl 10× ligase buffer (Section 2.8.5) and 1 µl T4 DNA ligase (New England Biolabs. 6 weiss units). The ligation mixture was incubated at 16°C overnight and analyzed afterwards by agarose gel electrophoresis in TBE buffer (Section 2.2.3).

2.2.7. Preparation and Transformation of *Escherichia coli* Competent Cells

Preparation

This method was adopted from Sambrook *et al.* (1989) with modification. *E.coli* strain DH5α was streaked from a -70°C frozen stock onto ϕb agar plate to get single colonies (Section2.2.1). A single colony was inoculated into 5 ml ϕb medium and incubated at 37°C with

shaking for 2 hours until OD_{600} reached about 0.3. The culture was poured into 100 ml ϕb medium and incubated with shaking for another 2–3 hours until OD_{600} reached 0.45. After that, the cells were kept on ice for 5 minutes and harvested by centrifugation at 5,000 rpm for 10 minutes at 4°C (Beckman High Speed Centrifuge JA 20 rotor). The cell pellet was resuspended in 40 ml Tfb I (Section 2.8.6) and then kept on ice for 5 minutes. The cell suspension was re-centrifuged again at 5,000 rpm for 10 minutes at 4°C. The cell pellet was resuspended in 4 ml Tfb II (Section 2.8.6) and kept on ice for 15 minutes afterwards. Then 200 µl bacterial cells was aliquoted into sterile microcentrifuge tubes and stored at -70°C.

Transformation

Prior to the transformation experiment, 200 µl competent cells were thawed on ice. 10 µl of transforming plasmid DNA was added to the competent cells and mixed gently. The tube was kept on ice for 30 minutes and then heat-shocked at 42°C for exactly 2 minutes for the DNA to enter the cell via the transient opening of the cell membrane. The tube was immediately returned to ice to chill the cells for 2 minutes.

Then 400 µl LB (Section 2.8.1) medium pre-heated to 37°C was added. The culture was incubated at 37°C for 50 minutes to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. 50 to 100 µl of the transformed cells were spreaded onto LB agar plate containing appropriate antibiotic and incubated at 37°C overnight. The transformed clones were screened by minipreparation of plasmid DNA (Section 2.2.8) and restriction enzyme digestion (Section 2.2.2).

2.2.8. Minipreparation of Plasmid DNA

This method was adopted from Sambrook *et al.* (1989) with minor modification. Liquid culture of bacterial strain was started as described in Section 2.2.1. Bacterial cells were spun down from 1.5 ml overnight culture in microcentrifuge tube at 13,000 rpm for 1 minute in a bench top microcentrifuge (Eppendorf). The supernatant was discarded and the last drop was removed with a fine pipette. The bacterial pellet was resuspended completely in 0.3 ml P1 (Section 2.8.7) containing 100 µg RNase/ml. 0.3 ml of P2 (Section 2.8.7) was subsequently added to lyse the cells. The content was mixed immediately but gently by inverting the

tube 4–6 times and the mixture was allowed to stand at room temperature for 5 minutes. Then 0.3 ml P3 (Section 2.8.7) was added and mixed in the same way to neutralize the lysate. Cell debris and chromosomal DNA were removed by centrifugation at 13,000 rpm for 15 minutes in the microcentrifuge. The supernatant was carefully pipetted out and transferred to a new microcentrifuge tube. 0.45 ml isopropanol was added to precipitate the DNA and the mixture was kept on ice for 15 minutes. The plasmid DNA was spun down at 13,000 rpm for 15 minutes and the pellet was washed with 0.5 ml 70% ethanol. After centrifugation at 13,000 rpm for another 10 minutes, the DNA pellet was dried in a 60°C oven and dissolved in H_2O at the desired concentration.

2.2.9. Preparation of Plasmid DNA using Magic™ Minipreps DNA Purification kit from Promega

This method follows the protocol supplied by the company. 3 ml of an overnight culture was centrifuged to harvest the bacterial cells. The cell pellet was resuspended in 200 µl P1 (Section 2.8.7) containing 100 µg RNase/ml. 200 µl P2 (Section 2.8.7) was then added and mixed by

inverting the tube several times until a clear lysate was observed. After that, 200 µl P3 (Section 2.8.7) was added and mixed in the same way to neutralize the lysate. Cell debris and chromosomal DNA were spun down at 13,000 rpm in a microcentrifuge for 5 minutes, and discarded. The cleared supernatant containing the DNA was decanted into a new microcentrifuge tube. 1 ml of Magic™ Minipreps DNA purification resin was added to the supernatant and mixed by inverting the tube for the DNA to adsorb onto the resin. A Magic™ Minicolumn was attached to a 3 ml disposable syringe barrel. The resin/DNA mixture was then pushed gently into the Minicolumn. The Minicolumn was washed with 2 ml Column Wash Solution and centrifuged at 13,000 rpm in a microcentrifuge for 20 seconds to dry the resin. 50 µl of preheated (65–70°C) water was applied to the Minicolumn. The DNA was eluted, by centrifugation at 13,000 rpm for 20 seconds in a microcentrifuge, into a fresh microcentrifuge tube attached to the outlet of the Minicolumn.

2.2.10. Preparation of Plasmid DNA using Qiagen-pack 100 Cartridge

The following method was adopted from the 3rd edition of the Qiagen-tips Protocol. Liquid culture of bacterial strain was started by inoculating single colony into 200 ml of liquid medium. (Section 2.2.1). Bacterial cells were harvested by centrifugation at 5,000 rpm for 10 minutes at 4°C (Beckman High Speed Centrifuge with JA 20 rotor). The cell pellet was resuspended in 8 ml P1 (Section 2.8.7) containing 100 μg RNase/ml. 8 ml P2 (Section 2.8.7) was subsequently added and mixed gently to lyse the cells, the mixture was allowed to stand at room temperature for 5 minutes. Then 8 ml P3 (Section 2.8.7) was added and gently mixed to neutralize the lysate. Cell debris and chromosomal DNA were removed by centrifugation at 12,000 rpm for 30 minutes at 4°C (Beckman JA 20 rotor). The supernatant was decanted into a fresh tube and re-centrifuged for another 10 minutes at 4°C to obtain a particle-free clear lysate of about 20 ml.

A QIAGEN-tip 100 (QIAGEN Inc.) was equilibrated with 3 ml QBT (Section 2.8.8) and allowed it to empty by gravity flow. 1/2 volume

(about 10 ml) of the prepared lysate was applied to the column, and the plasmid DNA was allowed to adsorb onto the resin by gravity flow. The column was then washed with 10 ml QC (Section 2.8.8). The plasmid DNA adsorbed onto the resin was finally eluted by 5 ml QF (Section 2.8.8). The above procedure was repeated to purify the plasmid DNA from the remaining lysate.

The two fractions of purified DNA was pooled together. The plasmid DNA was precipitated by 0.7 volume of isopropanol with incubation on ice for 15 minutes. The precipitated DNA was recovered by centrifugation at 13,000 rpm (JA 20 rotor) for 30 minutes at 4°C. The DNA pellet was washed with 1 ml 70% ethanol. After re-centrifugation for 15 minutes, the DNA pellet was dried in 60°C oven for 30 minutes. The DNA was dissolved in 250 µl H_2O and stored at -20°C .

2.2.11. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The Bio-Rad Protein II gel-casting mould was assembled based on the manufacturer's instructions.

Running gel (12%) was prepared as follows:

Components	20 ml	5 ml
Water	6.6ml	1.6ml
30% acrylamide	8.0ml	2.0ml
1.5M Tris (pH 8.8)	5.0ml	1.3ml
10% SDS	200µl	50µl
10% APS	200µl	50µl
TEMED	8µl	2µl

Running gel solution was poured into the gel apparatus to 2/3 full and 1 ml isopropanol was added on the top of the solution surface to remove air bubbles and to keep the solution away from atmospheric oxygen. The gel was allowed to polymerize for 15–30 minutes. After polymerization, isopropanol was discarded and the gap between the glass plates was rinsed with water and blotted dry with blotting paper.

Stacking gel (3%) was prepared as follows:

Components	6ml	2ml
Water	4.1ml	1.4ml
30% acrylamide	1.0ml	0.33ml
1.0M Tris (pH 6.8)	0.75ml	0.25ml
10% SDS	60µl	20µl
10% APS	60µl	20µl
TEMED	6µl	2µl

Stacking gel solution was poured on top of the running gel and the comb was inserted into the gel solution. The gel was allowed to polymerize for 15 minutes.

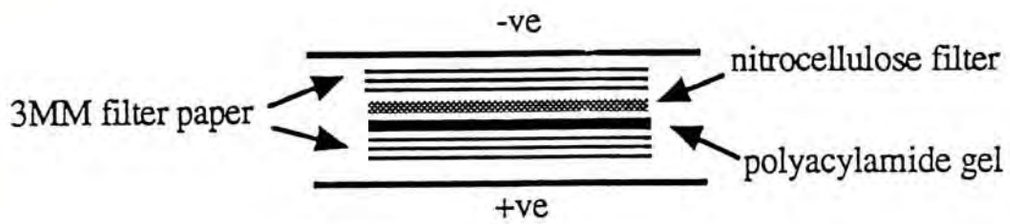
15 μl sample for Mini-gel and 30 μl sample for Large-gel was boiled with equal volume of 2× loading buffer (Section 2.8.9) for 3 minutes. The sample mixture was then applied into the wells. Electrophoresis was conducted at 35 mA for about 105 minutes (Large-gel) or 45 minutes (Mini-gel) until the bromophenol blue dye reached the bottom of the running gel. The gel was removed from the gel apparatus and the stacking gel was excised. The running gel containing the resolved proteins can either be stained with Coomassie Brilliant Blue or used to perform Western blotting.

Coomassie Brilliant Blue Staining

After electrophoresis, the running gel was immersed in staining solution (Section 2.8.9) for 30 minutes at 70°C or overnight at room temperature. Afterward, the gel was destained by soaking in destaining solution (Section 2.8.9) overnight. The destaining solution was renewed until the destaining process finished.

2.2.12. Western Blot detection of TCS

After electrophoresis, the stacking gel was excised. Six 3MM filter paper and a piece of nitrocellulose filter with the same size as the running gel was prepared. The nitrocellulose filter was soaked in distilled H_2O while the 3MM filter paper was soaked in Transfer buffer (Section 2.8.10). The semi-dry electroblotter (BioRad) was set up as follows:



Electroblotting was conducted for 1 hour at a constant current of 0.8 mA per cm^2 of the nitrocellulose filter. During this time, the proteins migrated from the gel towards the anode and attached to the nitrocellulose paper.

After electroblotting, the nitrocellulose filter was soaked in 10 ml TBSTM (Section 2.8.10) for 1 hour with gentle agitation on a platform shaker. This allows the remaining potential binding sites on the filter to be blocked with the irrelevant proteins. After that, the TBSTM was discarded and 10 ml of the primary rabbit antibody directed against TCS protein was added and the filter was swirled for 1 hour. Afterwards, the

filter was washed 3 times with TBST for 5 minutes. After the last wash, 10 ml of secondary goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (Section 2.8.10) was added and the filter was swirled again for 1 hour. Then, the filter was washed 3 times with TBST for 5 minutes. Chromogenic substrate mixture (Section 2.8.10) was prepared *in situ* and added to the filter. The filter was gently swirled for the colour to develop. When desired intensity of the colour was obtained, the reaction was stopped by rinsing the filter several times with distilled water. The filter was blotted dry against blotting paper or photographed to provide a permanent record of the experiment.

2.2.13. Polymerase Chain Reaction (PCR)

The following method was based on the 'Protocol For DNA Amplification' of the GeneAmp® PCR Reagent kit (Perkin Elmer Cetus). Primers used in the present study are listed in (Fig. 2.1).

1. Forward deletion primer of TCS (27 mer)
   GTTTTCCATATGAATCTGAGAAAAGCT

2. Reverse primer C of TCS (18 mer)
   TTGAGCAACAAATTGGGA

Fig 2.1 Primers utilized in Polymerase Chain Reaction

PCR was generally started by preparing the following reaction mixture, 5 µl 10× Reaction buffer, 4 µl of dNTP mix. (2.5 mM of dATP, dCTP, dGTP, dTTP), 2.5 µl of forward primer (20 µM) and 2.5 µl of reverse primer (20 µM) in a 500 µl microcentrifuge tube. Template DNA (about 100–200 ng) was added to the reaction mixture and the final volume was adjusted to 50 µl with water. In some of the experiments, the reaction mixture was incubated at 94°C for 5 minutes to denature the template DNA before 1 µl of AmpliTag® DNA Polymerase (5 Units/µl) was added. In other experiments, 1 µl DNA Polymerase was added directly to the reaction mixture. Finally, 50 µl of light mineral oil (Sigma M5904) was overlaid onto the reaction mixture to reduce evaporation or refluxing during the reaction. The PCR amplification was performed under the corresponding cycle of individual experiment, but generally including three subsequent steps (Denaturation, Annealing and Polymerization). After PCR amplification, 25 µl PCR product was carefully pipetted (avoiding the oil) and mixed with 5 µl 6× agarose gel loading buffer (Section 2.8.4). The PCR product mixture was then analyzed by 2% agarose gel electrophoresis in TBE buffer (Section 2.2.3).

2.3. Construction of the Plant Transformation Vectors

2.3.1. Construction of pSLJ 58210 (Fig. 2.2)

1.2 μg of pET 58210 DNA and 1.25 μg of pSLJ 4D4 DNA were digested with restriction enzymes *Bam*H I and *Nco* I in NEBuffer 3 and 100 μg BSA/ml. After agarose gel electrophoresis in TAE buffer (Section 2.2.3), the 0.75-kb fragment from pET 58210 and the 5.3-kb fragment from pSLJ 4D4 were purified using GeneClean® kit and dissolved in 15 μl H_2O. 5 μl of the 0.75-kb fragment, 2.5 μl of the 5.3-kb fragment, 0.5 μl of water, 1 μl 10× T4 DNA ligation buffer and 1 μl of T4 DNA ligase were mixed together and incubated at 16°C overnight (Section 2.2.6). The ligation mixture was transformed into *E.coli* strain DH5α as described in Section 2.2.7. The transformed colonies were screened by minipreparation (Section 2.2.8) and restriction enzyme digestion with *Bam*H I and *Nco* I (Section 2.2.2). The ligated pSLJ 58210 was then prepared by Qiagen-pack 100 Cartridge (Section 2.2.10).

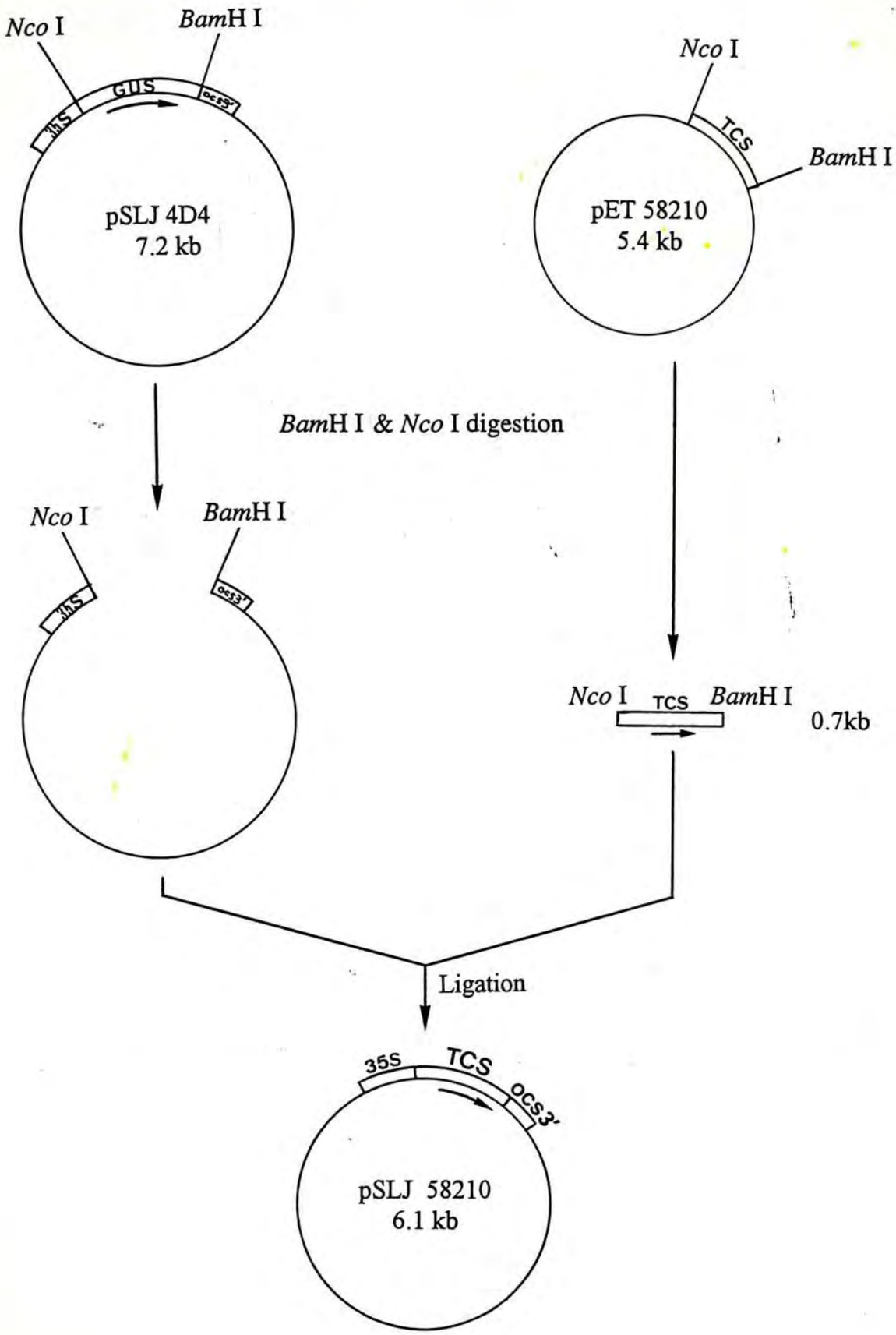


Fig. 2.2 Construction of pSLJ 58210

2.3.2. Construction of pSLJ TCS1 and pSLJ TCS2 (Figs. 2.3A & B)

5 μg of pSLJ 58210 was digested with restriction enzymes *Bgl* II and *Hin*d III in NEBuffer 2. 1 μg of pSLJ 44024 and pSLJ 44026 were digested with restriction enzymes *Bam*H I and *Hin*d III in NEBuffer 2 with 100 μg BSA/ml respectively. After agarose gel electrophoresis, the 1.75-kb fragment from pSLJ 58210 was purified by GeneClean® kit and dissolved in 20 μl H_2O. The 26-kb DNA from pSLJ 44024 and pSLJ 44026 were purified by Phenol/Chloroform extraction (Section 2.2.5) and finally dissolved in 20 μl H_2O. 6.5 μl of the 1.75-kb fragment, 14 μl of the 26-kb fragment, 2.5 μl 10X T4 DNA ligation buffer and 2 μl T4 DNA ligase were mixed together and incubated at 16°C overnight (Section 2.2.6). The ligation mixture was transformed into *E.coli* DH5α as described in Section 2.2.7. The transformed colonies were screened by minipreparation (Section 2.2.8) and restriction enzyme digestion with *Xho* I in NEBuffer 2 with 100 μg BSA/ml (Section 2.2.2). The ligated pSLJ TCS1 and pSLJ TCS2 were then prepared by Qiagen-pack 100 Cartridge (Section 2.2.10).

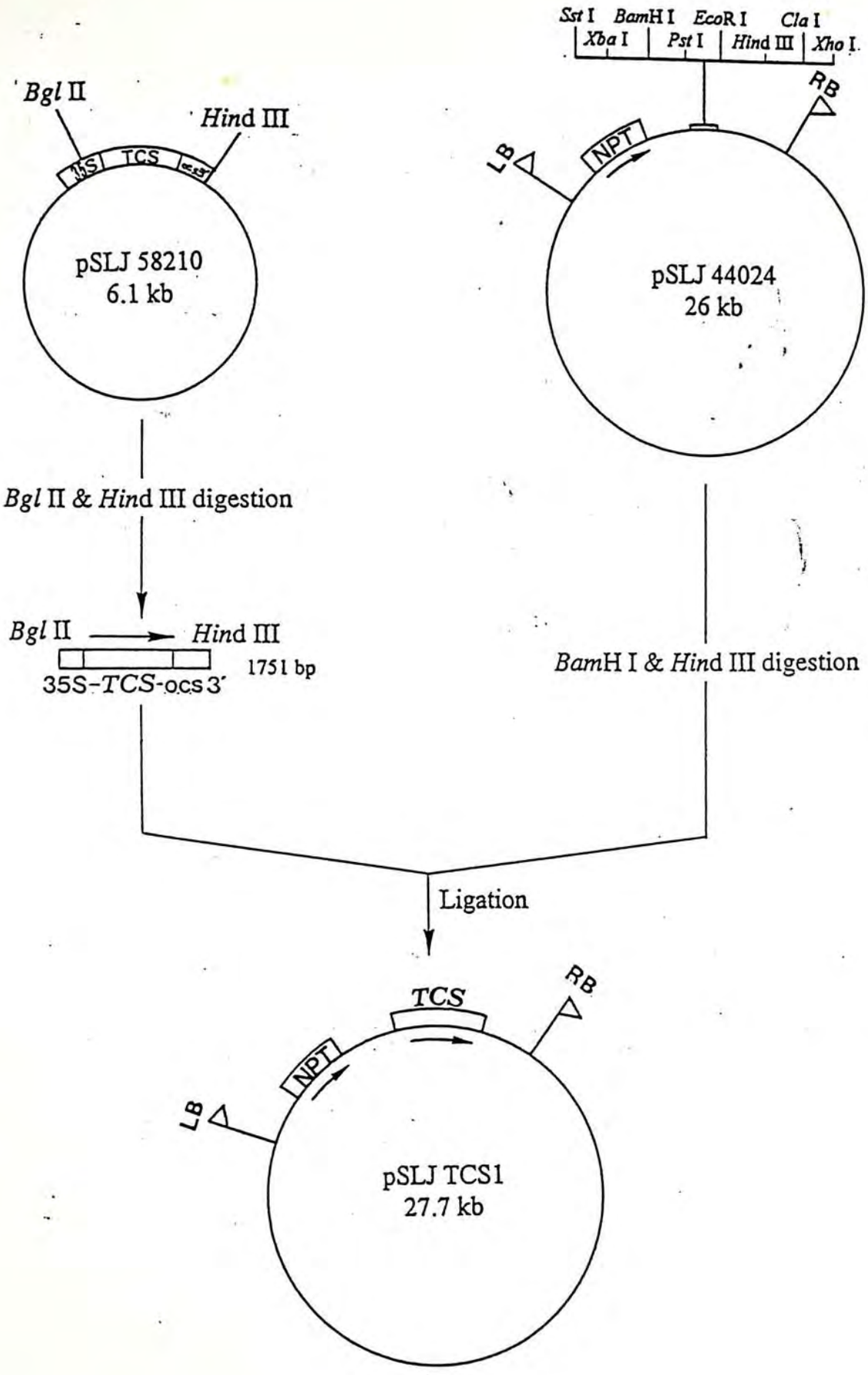


Fig. 2.3A Construction of pSLJ TCS1

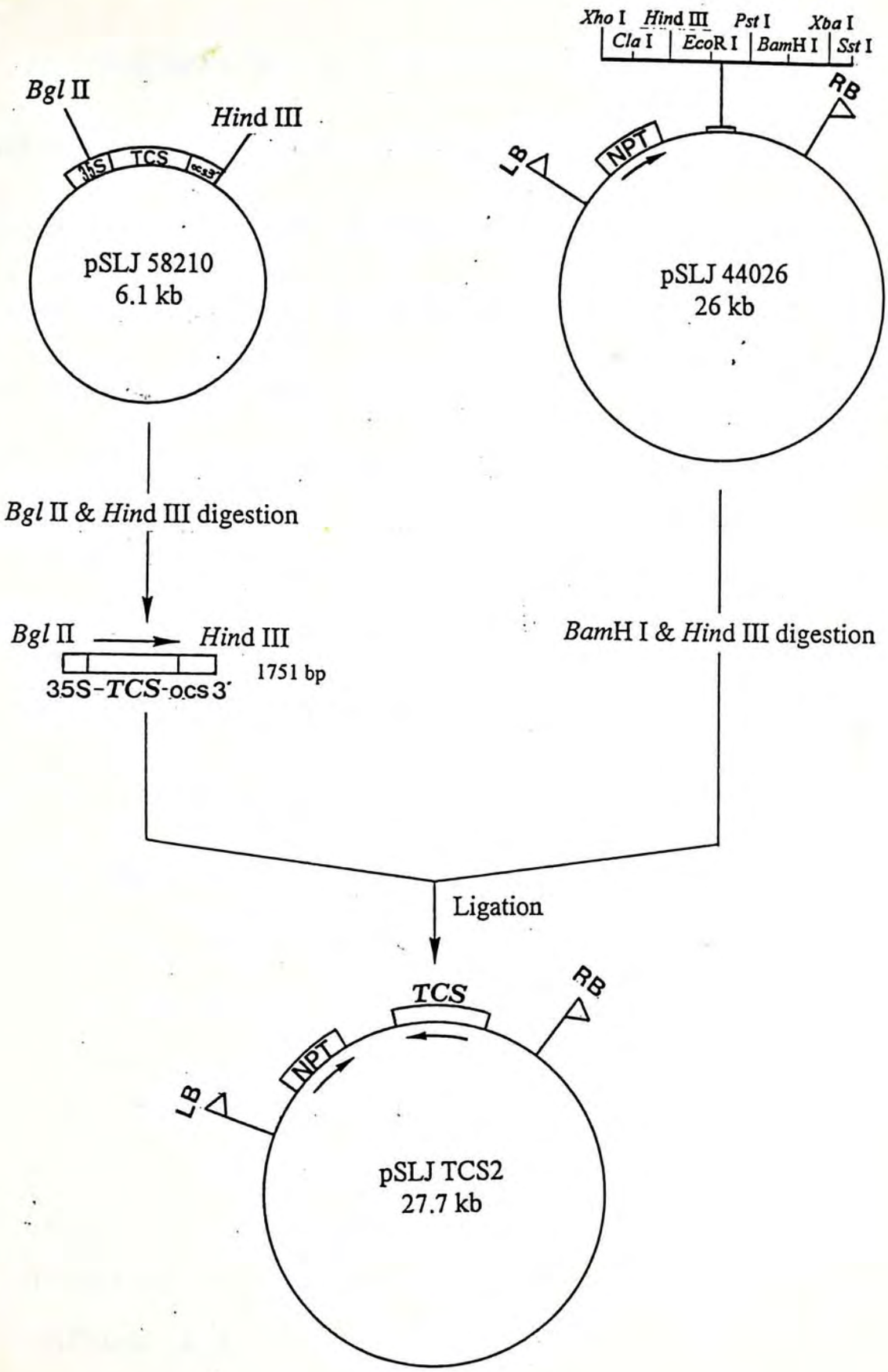


Fig. 2.3B Construction of pSLJ TCS2

2.3.3. Conjugation of pSLJ TCS1 and pSLJ TCS2 into *A.tumefaciens* by Triparental Mating

Liquid culture of *E.coli* strain DH5α (pSLJ TCS1) was grown up in 5 ml LB medium containing 12.5 μg Tetracycline/ml with shaking at 37°C as described in Section 2.2.1. *E.coli* strain HB101 (pRK 2013) was also grown up in LB medium with 25 μg Kanamycin/ml under the same condition. *Agrobacterium tumefaciens* LBA4404 was grown up in 5 ml YEP medium with 0.2% glucose at 28°C with shaking (Section 2.2.1). After 24 hours, the growing *E.coli* strains were spun down at 13,000 rpm for 1 minute and resuspended in the same volume of LB. 200 μl of each of the three bacterial strains were used to set up the following mixture in microcentrifuge tubes:

1. DH5α (pSLJ TCS1) or DH5α (pSLJ TCS2)
2. HB101 (pRK 2013)
3. LBA4404
4. DH5α (pSLJ TCS1) or DH5α (pSLJ TCS2) + HB101 (pRK 2013)
5. DH5α (pSLJ TCS1) or DH5α (pSLJ TCS2) + LBA4404
6. HB101 (pRK 2013) + LBA4404
7. HB101 (pRK 2013) + LBA4404 + DH5α (pSLJ TCS1) or DH5α (pSLJ TCS2)

Then, 200 µl from each of the bacterial mixtures were spread onto LB agar plates (no antibiotic selection) and incubated with the plates upside-up at 30°C. After 24 hours, a 'loopful' of cells were removed from each plate and streaked onto Min T agar plates (Section 2.8.1) containing 50 µg Kanamycin/ml and 500 µg Streptomycin/ml and then incubated at 28°C overnight. Single colonies were restreaked on two more occasions in the following few days.

Screening of Transconjugated colonies

Plasmid DNA was isolated from transformed colonies by Magic minipreparation (Section 2.2.9). The size of the plasmids were analyzed by agarose gel electrophoresis with the pSLJ 44024 or pSLJ 44026 as a marker. The plasmids were also analyzed by restriction enzyme digestion with *Xho* I in NEBuffer 2 and 100 µg BSA/ml (Section 2.2.2) and by PCR using primer C and forward deletion primer of TCS (Fig. 2.1). 100 ng miniprep plasmids from putative transconjugants were used as templates and the reaction mixture was prepared as decribed in Section 2.2.13.

The DNA amplification was performed under the following condition:

Cycle	Denaturation	Annealing	Polymerization
35	1 min. at 94°C	1 min. at 55°C	1 min. at 72°C

2.4. Transformation of Tobacco Leaf Explants by *Agrobacterium tumefaciens*

2.4.1. Growth of *A.tumefaciens* LBA4404 (pSLJ TCS1)

Liquid culture of LBA4404 (pSLJ TCS1) was prepared as described in Section 2.2.1 in Min T medium containing 50 μg Kanamycin/ml and 500 μg Streptomycin/ml, and were incubated overnight at 28°C with shaking. Just before the inoculation experiment, 400 μl of the overnight culture was added to 20 ml Min T medium to make a 1:50 dilution.

2.4.2. Surface Sterilization of tobacco leaves

Young (just fully expanded) leaves of tobacco (*Nicotiana tabacum* var. Wisconsin 38) were placed in 500 ml sterile distilled water containing 10% Chlorox and 0.1% Tween 20 for 20 minutes with gentle stirring.

The leaves were subsequently rinsed thoroughly with sterile distilled water for 10 minutes and the rinse was repeated twice.

2.4.3. Inoculation of tobacco leaf explants with *A.tumefaciens* LBA4404 (pSLJ TCS1)

Surface sterilized tobacco leaves were dissected to remove mid-rib and lateral-rib, and were then excised into 1 cm^2 leaf explants. 10 leaf explants were then exposed to LBA4404 (pSLJ TCS1) by dipping them for 10 minutes in 20 ml of the *Agrobacterium* suspension (Section 2.4.1).

After soaking in the bacterial suspension, leaf explants were blotted dry on sterile filter paper and placed upside down on solid culture medium containing MS salts and vitamins (Section 2.8.2), 30 g/l sucrose, 1 mg BA/l, 0.1 mg NAA/l and 0.8% agar at pH 5.8. The plates were wrapped with parafilm (Whatman) and incubated for 3 days at room condition.

2.4.4. Regeneration of shoots from Transformed explants

After incubation for 3 days, explants were transferred to shoot induction and selective medium (the same as above) with 500 μg Carbenicillin/ml and 100 μg Kanamycin/ml and moved to 24°C, 16/8 light/dark cycle from fluorescent lamps. Explants were subcultured on the same selective medium at 2 weeks intervals.

2.4.5. Rooting of Transformed shoots

Shoots regenerated from transformed explants were excised and trimmed to discard the leaves at the base of the shoots. The trimmed shoots were then rapidly transferred to the root induction medium [MS salt and vitamins (Section 2.8.2), 3 g/l sucrose, 0.6% agar, pH5.8] with 250 μg Carbenicillin/ml and 100 μg Kanamycin/ml, and grown under the same condition for the shoot induction.

2.4.6. Re-establishment of cultured Plantlets in soil

Rooted transformed shoots were gently removed from the agar. Remaining agar sticking to the roots was carefully washed off under running tap water. The plantlets were then placed in an appropriately

sized hole in a soil-filled plant pot. The roots were covered with soil and pressed gently to support the plantlets. The potted plantlets were sprayed with water to moisten the soil and covered with clear cling film to maintain a humid environment as soon as possible. The plantlets were grown under controlled environment with 14 hours photoperiod, 22°C/18°C day/night cycle from a combination of incandescent and fluorescent lamps.

After one week, the film was opened for 5 minutes and watering was allowed. This period was increased from 5 minutes to 5 hours over the following two weeks. Finally, the film was removed and a nutrient supplementation of 20N-20P-20K (Hyponex® no.2) was provided at weekly intervals.

2.5. Analysis of the Regenerated Transgenic Tobacco

2.5.1. Isolation of plant leaf protein

Fresh leaf was excised from transgenic tobacco at several leaves stage. The leaf was dissected to remove the mid-rib, 0.2 g of leaf material was

frozen with liquid nitrogen in a microcentrifuge tube and ground into powder with a sealed-end pipet tip.

Powdered plant material was incubated in 200 µl extraction buffer (50 mM HEPES at pH 7.0, 5 mM β-mercaptoethanol and 0.8 mM PMSF) for 20 minutes. The tube was inverted occasionally to keep the extract mixed during the incubation. Afterwards, the debris was spun down at 13,000 rpm for 10 minutes in a microcentrifuge. The supernatant containing the extracted proteins was saved and stored at -20°C.

Protein assay (Bradford's method)

This method was adopted from Bradford (1976) and performed by using Protein assay dye reagent (BioRad). BSA solutions containing 5 to 50 µg/ml protein were prepared by serial dilution in H_2O. Meanwhile, 1/100 dilution of the protein extracts obtained from the transgenic and the wild type tobacco plants were also prepared. After that, 0.8 ml of each protein dilution was pipetted into microcentrifuge tubes in duplicate. 0.2 ml of the dye reagent (BioRad) was then added to the tubes and the contents were mixed by inversion. The absorbance at 595 nm was

measured after 5 minutes against a reagent blank prepared from 0.8 ml water and 0.2 ml of the dye reagent. The absorbance was plotted against the weight of the BSA protein resulting in a standard curve used to determine the yield of protein extracted from the trangenic and the wild type tobacco plants.

2.5.2. SDS-PAGE and Western blot detection of TCS

Aliquots corresponding to 30 μg of total leaf proteins determined by the Protein assay were analyzed by SDS-PAGE and Western blotting for the expression of TCS as described in Section 2.2.11 and 2.2.12.

2.5.3. Anti-viral assay of Transgenic tobacco against TuMV

Transgenic tobacco plants which had been shown positive by Western blotting were used in the anti-viral assay. 1/500 dilution of purified TuMV was mechanically inoculated onto a pair of the a pair of the opposite leaves of three transgenic plants (Section 2.6.3). The same virus solution was also rubbed onto two opposite leaves of three wild type

tobacco plants as the control. The results were monitored by observing the appearance of local lesion on the treated leaves.

2.6. Bioassay of Inhibitory activity of TCS protein against TuMV

2.6.1. Preparation of biologically active TCS protein

Expression of recombinant TCS in *Escherichia coli*

Expression host *E.coli* BL21 (DE3, pLysS) was transformed by pET 58210. Preparation of competent cells and transformation were described in Section 2.2.7.

A single transformed colony of BL21 (DE3, pLysS pET 58210) was grown overnight in 5 ml M9ZB (Section 2.8.1) containing 50 μg Ampicillin/ml and 25 μg Chloramphenicol/ml at 37°C with shaking (Section 2.2.1). It was followed by 1% inoculation in 500 ml fresh M9ZB medium containing the same antibiotics as described above. The cell culture was grown at 37°C until the cell density reached OD_{600} = 0.8 (about 4–5 hours). IPTG was added to 0.4 mM and the cells were

cultured at 37°C for another 3 hours. The cells were harvested by centrifugation at 3000×g for 10 minutes at 4°C (Beckman JA 12-2 rotor) and the weight of the pelleted cells was measured.

Purification of recombinant TCS from induced *E.coli*

This method was adopted from Zhu *et al.* (1991) with minor modification. 4 g of cell pellet was resuspended in 15 ml 0.05 M sodium dihydrogen phosphate (pH 6.5) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) as protease inhibitor and 0.1% β-mercaptoethanol as antioxidant. The cells were lysed by sonication for 8 cycles of 30 seconds at 20W output at 4°C and to release the TCS. The lysate was centrifuged at 21000×g for 30 minutes at 4°C to remove the insoluble cell debris. The soluble fraction containing TCS was dialysed overnight against 2 litre of 0.05 M sodium dihydrogen phosphate (pH 6.5) (buffer A) to equilibrate the TCS, and was then loaded onto a column no.1 (1.6 × 19 cm) of CM-Sepharose (Pharmacia) already equilibrated with the same buffer at a flow rate of 15 ml/hr. The eluate was collected in 2 ml fractions and the column was washed with buffer A (about 100 ml) to remove the unadsorbed components until the OD_{280} was less than

0.1. Then a step gradient of 0.5 M sodium chloride in buffer A (about 100 ml) was applied to elute all the absorbed proteins including TCS. Fractions ($OD_{280} > 0.2$) were pooled and dialysed against 1 litre buffer A overnight at 4°C. At the same time, the column was equilibrated with 200 ml of buffer A overnight at a flow rate of 15 ml/hr. After dialysis, the solution was then filtered through 0.45μm millipore filter to remove the precipitate formed during dialysis and the filtrate containing TCS was then loaded onto the CM-Sepharose column no.2 (1.6 × 19 cm). The column was washed with 100 ml of buffer A until $OD_{280} < 0.1$, a linear gradient of NaCl (0 – 0.3 M of about 100 ml) was then applied to elute the TCS. Fractions ($OD_{280} > 0.2$) was pooled and dialysed against distilled water for 4 hours at 4°C to remove the salt. The dialysate was lyophilized for storage at -20°C.

2.6.2. Purification of TuMV from infected plant leaves

TuMV strain cabbage black ringspot (PV 17/FD) had been purchased from American Type Culture Collection, the virus was propagated in host plant *B.chinensis* by mechanical inoculation (Section 2.6.3) and mosaic symptom expressed on young leaves at about 2 weeks after virus

inoculation. Procedures for purification of TuMV was adopted from Lisa *et al.* (1981). To purify TuMV, the virus-infected leaves (~120 g) were harvested. The infected leaves were homogenized in a blender with extraction buffer (pH 8.5) containing 240 ml 0.5 M K_2HPO_4, 0.02 M Na_2SO_3 (reducing agent), 0.005 M Na_2EDTA and 0.01 M sodium diethyldithiocarbamate (DIECA) as chelating agents at 4°C. These agents were used to facilitate virus extraction and maintain the stability of virus. The resulting slurry was then emulsified with 240 ml Freon 113 and homogenized again in the blender. Then the virus was isolated by differential centrifugation in which the cell debris was spun down at 5,000 rpm (Beckman JA 20 rotor). The supernatant was spun again at 25,000 rpm (Beckman 45 Ti rotor). The pellet enriched with virus was resuspended in 5 ml of 0.05 M citrate buffer (pH 7.5) and mixed with CsCl to a density of 1 g/ml and centrifuged at 26,000 rpm (Beckman 45 Ti rotor) for 18 hours at 10°C. During isopynic (density gradient) centrifugation in CsCl, purified virus formed a single board band in the centrifuge tube and the tube was punctured to collect the virus.

2.6.3. Mechanical Inoculation of virus onto host plant

Inoculum was prepared by making a desired dilution of the purified virus in 0.05 M phosphate buffer (pH 7.5) with 10 mM sodium sulphite. The virus inoculum was rubbed onto the leaves which had been dusted with Celite as abrasive particles (Fig. 2.4). The treated plants were then placed under dim condition for two days to avoid desiccation of the wound leaves.

Fig. 2.4 Mechanical inoculation of TuMV to *N.tabacum*

2.6.4. Anti-viral assay on Local Lesion host

The anti-viral effect of TCS protein was assayed by the half leaf method using the local lesion host (*Nicotiana tabacum* var. Wisconsin 38) of TuMV. 1/250 dilution of purified TuMV was mixed 1:1 (v/v) with TCS protein or with water as control. The final concentrations of TCS protein were 250 μg/ml and 400 μg/ml. 100 μl TuMV/TCS inoculums were mechanically inoculated onto the right half of the leaf. While the TuMV/water inoculum was rubbed onto the opposite half leaf as the control. Each treatment was replicated on 10 leaves of 10 individual plants. The number of local lesions appeared were counted 7 days after inoculation and the percentage inhibition was calculated according to the following formula:

Percentage Inhibition = $(1 - T/C) \times 100$

C = the number of local lesions appeared on the control half leaf

T = the number of local lesions on the treated half

2.6.5. Anti-viral assay on Systemic host

The inhibitory effect of TCS protein on TuMV was assayed using the systemic host *(Brassica parachinensis* var. 80 day). 200 μl of TCS protein was applied at concentration of 1, 10, 100 μg/ml with a paintbrush to the expanded young leaf. Each treatment was replicated on a pair of the opposite leaves of each of 10 individual plants. The treatment was repeated with water as the control. After 24 hours of treatment, 50 μl of 1/100 dilution of purified TuMV was mechanically inoculated onto all the TCS treated leaves and the control leaves. The results were monitored by observing the expression of mosaic symptom on the young leaves in the following 2 weeks. Finally, the percentage of plants showing virus symptom was plotted against the day of expression.

2.7. Establishment of the plant culture medium for efficient Regeneration from tissue explants of *Brassica parachinensis*

2.7.1. Preparation and Sterilization of the culture medium

The basal medium used in the present study is the Murashige and Skoog (MS) basal salt mixture (Section 2.8.2) supplemented with the Murashige and Skoog vitamins (Section 2.8.2) (Murashige and Skoog, 1962). The plant tissue culture medium was generally prepared in a mixture of basal salts, vitamins, sucrose and heat-stable growth regulators. While stirring, the medium was adjusted to pH 5.7–5.8 using NaOH or HCl. For solid medium, 0.6–0.8% agarose was added. The medium was then sterilized by autoclaving at 121°C and 15–20 psi for 15 minutes.

Some components such as antibiotics and silver nitrate were thermolabile. Stock solutions of the heat-labile components were prepared and filter-sterilized through a 0.22 μm filter into sterile

containers. The filtered solution was aseptically added to the autoclaved culture medium which had been cooled to approximately 35–45°C. The medium was then dispensed into culture vessels under sterile condition.

2.7.2. Preparation of Sterile seedings of *B.parachinensis*

Surface sterilization of seeds

Seeds of *B.parachinensis* var. 80 day were surface-sterilized with 10% Chlorox and 0.1% Tween 20 in distilled water for 20 minutes with stirring. After sterilization, the seeds were rinsed with sterile distilled water for 10 minutes with stirring. Distilled water was poured off and the rinse was repeated twice.

Growth of sterile seedings

Surface sterilized seeds were plated on Murashige and Skoog basal medium with 3% sucrose and 0.6% agarose in a culture box. The culture was incubated at around 24°C, 16/8 light/dark cycle from fluorescent lamps to allow the seeds to germinate and develop into seedlings under sterile condition.

2.7.3. Regeneration from Cotyledon petiole and Hypocotyl segment explants

Cotyledon petiole culture

Fully expanded cotyledon petioles were excised from 7-day-old sterile seedlings (Fig 2.5). The explants were cultured on MS basal medium (Section 2.8.2) containing 0.8% agarose, 2% sucrose, silver nitrate at 5 mg/1 and Benzylaminopurine (BA) at concentration ranged from 0.5 to 5 mg/l at pH 5.7. The culture was incubated at around 24°C, 16/8 light/dark cycle from fluorescent lamps.

Hypocotyl segment culture

5–7mm hypocotyl segments were excised from 7-day-old sterile seedlings (Fig. 2.5). The explants were cultured on MS basal medium (Section 2.8.2) containing 0.8% agarose, 2% sucrose, silver nitrate at 5 mg/l, Naphthaleneacetic acid (NAA) at 0.5 mg/l and BA at concentration of 2, 4, 6 or 10 mg/l at pH 5.7. The culture was incubated under the same condition for the culture of cotyledon petiole.

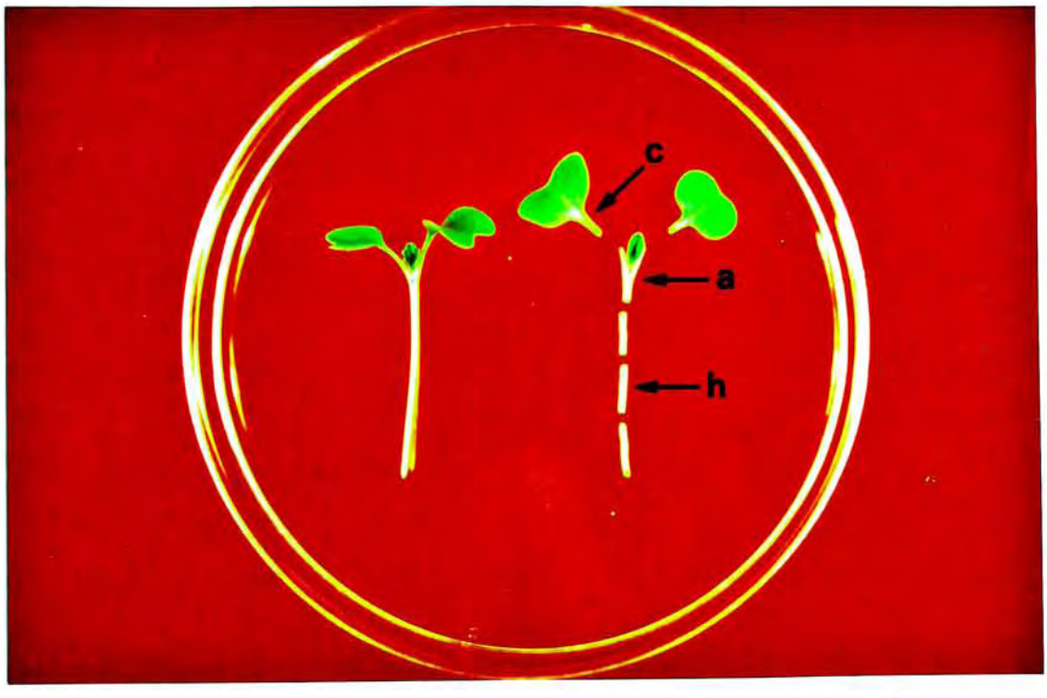


Fig. 2.5 Cotyledon petioles (c), hypocotyl segments (h) and the shoot apex (a) excised from the sterile seedling of *B.parachinensis*.

2.7.4. Regeneration from Internode stem segment explants of shoot culture

Shoot apex (Fig. 2.5) excised from the 7-day-old sterile seedlings were cultured on MS basal medium (Section 2.8.2) containing 0.8% agarose and 3% sucrose. The shoot culture was incubated under the same condition for the growth of sterile seedlings in Section 2.7.2.

Internode stem segment culture

After 5–6 weeks of culture, 3–5 mm stem segments were excised from the internode regions of the shoot culture and were cultured on MS basal medium (Section 2.8.2) containing 0.8% agarose, 2% sucrose, 5 mg BA/l, 0.5 mg NAA/l and 5 mg silver nitrate/l at pH 5.7. The culture was incubated under the same condition mentioned for the culture of cotyledon petioles and hypocotyl segment explants.

2.8. Reagents and Buffer

2.8.1. Media for Bacterial culture

LB (Luria-Bertani medium)

1% Tryptone

0.5% Yeast extract

1% NaCl

pH 7.2, autoclaved at 121°C for 15 minutes

ϕb medium

2% Tryptone

0.5% Yeast extract

1% $MgSO_4 \cdot 7H_2O$

pH 7.2, autoclaved at 121°C for 15 minutes

YEP medium

1% Peptone

1% Yeast extract

0.5% NaCl

pH 7.2, autoclaved at 121°C for 15 minutes

Min T (Minimal T Medium)

20 × T salts	50ml
20 × T buffer	50 ml
20% Sucrose	25 ml

To 1 litre with distilled water

20 × T salts:	NH_4Cl	20g
	$MgSO_4 \cdot 7H_2O$	4g
	$MnCl_2$	0.04g
	$CaCl_2$	0.2g
	$FeSO_4 \cdot 7H_2O$	0.1g

To 1 litre with distilled water

20 × T Buffer:	K_2HPO_4	210g
	KH_2PO_4	90g

To 1 litre with distilled water

Solid media contain 1.5 % agar

autoclaved at 121°C for 15 minutes

M9ZB medium

1g NH_4Cl

3g KH_2PO_4

15.2g Na_2PO_4

10g Trypone

5g NaCl

To 1 litre with distilled water

Just before use, the followings were added:

20 ml 20% glucose (filter-sterilized)

1 ml 1M $MgSO_4$ (filter-sterilized)

Antibiotics:

Ampicillin	(Ap)	(Sigma A-9518)
Chloramphenicol	(Cm)	(Sigma C-3078)
Kanamycin	(Km)	(Sigma K-4000)
Streptomycin	(Sm)	(Glaxo Lot.B-3790C3KA)
Tetracycline	(Tc)	(Oxoid Lot.30283)

2.8.2. Media for Plant tissue culture

Murashige and Skoog Modified Basal Salt Mixture

" Finer and Nagasawa Modification" (Sigma M-8775)

Components	mg/l
Ammonium Nitrate	825.0
Boric Acid	6.20
Calcium Chloride Anhydrous	332.20
Cobalt Chloride Hexahydrate	0.0250
Cupric Sulfate Pentahydrate	0.0250
Disodium EDTA Dihydrate	37.260
Ferrous Sulfate Heptahydrate	27.80
Magnesium Sulfate Anhydrous	180.70
Manganese Sulfate Monohydrate	16.90
Potassium Iodide	0.830
Potassium Nitrate	3030.0
Potassium Phosphate Monobasic	170.0
Sodium Molybdate Dihydrate	0.250
Zinc Sulfate Heptahydrate	8.60

Murashige and Skoog Modified Vitamin Powder (1000X)

(Sigma M-6896)

Components	**mg/l**
Glycine	2.0
myo-Inositol	100.0
Nicotinic Acid	0.5
Pyridoxine-HCl	0.5
Thiamine-HCl	1.0

Growth Regulators:

BA (Benzylaminopurine) (Sigma B-9395)

NAA (Naphthaleneacetic acid) (Sigma N-0640)

Antibiotics:

Carbenicillin (Sigma C-3416)

Kanamycin (Sigma K-4000)

2.8.3. Restriction Enzymes

*Bam*H I (Biolabs 20 U/μ l)

Bgl II (Biolabs 8 U/μ l)

Hind III (Biolabs 20 U/μ l)

Nco I (Promega 6 U/μ l)

Xho I (Biolabs 20 U/μ l)

2.8.4. Buffers for Agarose Gel Electrophoresis

TAE (Tris-acetate), 1×

4.84 g/l Tris-base (pH8.0)

0.34 g/l Na_2EDTA

20mM Acetic acid

TBE (Tris-borate), 1×

10.9 g/l Tris-base (pH 8.2)

0.68 g/l Na_2EDTA

5.56 g/l Boric acid

6× Agarose gel loading buffer

0.25% Bromophenol blue

40% (w/v) Sucrose in water

2.8.5. DNA Ligation Buffer

5× ligase buffer

39.96 g/l Tris-base (pH 7.5)

2.38 g/l $MgCl_2$

3.86 g/l Dithiothreitol

5.09 g/l ATP

2.8.6. Reagents for preparation of *E.coli* competent cells

Tfb I

2.94 g/l KAc

12.09 g/l RbCl

1.11 g/l $CaCl_2$

6.3 g/l $MnCl_2$

15% Glycerol

Tfb II

2.09 g/l MOPS

8.33 g/l $CaCl_2$

1.56 g/l $RbCl_2$

15% Glycerol

pH adjusted to 6.5 with KOH

2.8.7. Reagents for preparation of Plasmid DNA

P1

6.06 g/l	Tris-base (pH 7.5)
3.36 g/l	Na_2EDTA
100µg	RNase A for 1ml buffer

P2

8 g/l	NaOH
1%	SDS

P3

250.06 g/l	KAc (pH 4.8)

2.8.8. Reagents for Qiagen-pack 100 Cartridge

QBT

43.83 g/l	NaCl
10.47 g/l	MOPS
15%	Ethanol
0.15%	Triton X-100
pH 7.0	

QC

73.05 g/l	NaCl
10.47 g/l	MOPS
15%	Ethanol
pH 7.0	

QF

1.25M	NaCl
50mM	MOPS
15%	Ethanol
pH 8.2	

2.8.9. Reagents for SDS-PAGE

2× SDS gel-loading buffer

1ml	1.0M Tris-Cl pH 6.8
4ml	10% SDS
2ml	Glycerol
0.02g	Bromophenol blue
0.2ml	β-mercaptoethanol

To 10 ml with distilled water

Staining solution

0.575g	Coomassie brilliant blue R-250
1.25ml	Ethanol
40ml	Acetic acid

To 400 ml with distilled water

Destaining solution

500ml	Ethanol
160ml	Acetic acid

To 2 litres with water

2.8.10. Reagents for Western blotting

Transfer buffer

5.81 g/l	Tris-base
2.93 g/l	Glycine
0.37 g/l	SDS
20% (v/v)	methanol

TBST (Tris-buffered saline – Tween)

1.211g	Tris-base
8.76g	NaCl
0.5ml	Tween-20

To 1 litre with distilled water and pH adjusted to 8.0

TBSTM (TBST – Milk)

100ml	TBST
3g	Milk powder

Dissolved by heating in microwave oven

Primary antibody solution (titer 1:5,000)

15ml	TBSTM
0.03%	NaN_3
30µl	Rabbit anti-TCS serum (home-made)

Secondary antibody solution (titer 1:7,000)

15ml	TBSTM
0.03%	NaN_3
2 µl	Goat anti-rabbit IgG (Promega)

AP (Alkaline phosphatase) buffer

12.11 g/l	Tris-base (pH 9.5)
5.84 g/l	NaCl
0.48 g/l	$MgCl_2$

Chromogenic substrate mixture

10ml	AP buffer
66µl	NBT (Nitroblue tetrazolium)
33µl	BCIP (5-bromo-4-chloro-3-indolylphosphate)

It is light-sensitive and should be prepared *in situ*.

Chapter 3
Construction
of
Plant Transformation Vectors

Chapter 3 Construction of Plant Transformation Vectors

3.1. Introduction

For expression in plant cells the TCS encoding cDNA needs a suitable promoter and a 3′ terminator sequence to ensure efficient transcription, stability and translation of mRNA. In the present study, the promoter of the 35S gene (P35S) of the Cauliflower mosaic virus (CaMV) and the 3′ polyadenylation sequence of the octopine synthase gene (ocs3′) of *Agrobacterium* were selected to construct the chimeric TCS encoding cDNA. The 35S promoter is a constitutive promoter and allows the expression of heterologous gene in plants. To achieve this, the 0.75-kb fragment of TCS cDNA was subcloned into the pSLJ 4D4 DNA to create the vector pSLJ 58210 (Fig. 2.2). Then, the chimeric construct 5′35S-TCS-ocs3′ was subcloned into the binary vectors pSLJ 44024 and pSLJ 44026 (Fig. 2.3A & B). These two binary vector plasmids differ from each other only by the opposite orientation of the pdBS polyLinker (the multi-cloning site).

After subcloning, two binary vector constructs pSLJ TCS1 and pSLJ TCS2 were obtained in which the chimeric TCS encoding cDNA was aligned to transcribe parallel to or convergent with the direction of transcription of the antibiotic resistance gene neomycin phosphotransferase.

Triparental mating was used to introduce the two binary vector constructs into *A.tumefaciens* LBA 4404. During the mating process, the pSLJ TCS1 or pSLJ TCS2 were firstly introduced into *E.coli* HB101 (pRK 2013) by conjugation. Then, the pRK 2013 DNA provided mobilization (*mob)* and transfer (*tra*) functions in *trans* to mobilize the pSLJ TCS1 DNA or pSLJ TCS2 DNA into *A.tumefaciens* LBA 4404.

3.2. Results

3.2.1. Construction of pSLJ 58210

Subcloning

The strategy of construction is shown in Fig. 2.2 and methods are described in Section 2.3.1. In the process, pET 58210 DNA and

pSLJ 4D4 DNA were digested respectively with restriction enzymes *Bam*H I and *Nco* I and all of the digestion products were loaded into a 1% agarose gel in TAE buffer. After electrophoresis, the 0.75-kb fragment cleaved from pET 58210 and representing the TCS cDNA, cleaved from pET 58210 and the 5.3-kb fragment excised from pSLJ 4D4 were purified by GeneClean® kit (Fig. 3.1.A). Afterwards, the 0.75-kb fragments of TCS cDNA were ligated to the 5.3-kb fragments of pSLJ 4D4 to construct the recombinant plasmid pSLJ 58210. The ligated DNA was then transformed into 50 µl *Escherichia coli* strain DH5α and two transformants were obtained.

Screening of the transformed colonies

The transformed colonies, labelled colony number 1 and 2 were screened by minipreparation and restriction enzyme digestion. The miniprep recombinant plasmid pSLJ 58210, resulted from the ligation between the 0.75-kb fragment of TCS cDNA and the 5.3-kb fragment of pSLJ 4D4, would have the expected size of about 6.1-kb. Also, pSLJ 58210 (6.1-kb) have a greater mobility as compared to pSLJ 4D4 (7.2-kb) in 1% agarose gel as shown in Fig. 3.1B.

Fig. 3.1A Analysis of isolated fragment (0.75-kb) of TCS cDNA and fragment (5.3-kb) of pSLJ 4D4 by 1% agarose gel electrophoresis.
Lane 1: 0.75-kb fragment of TCS cDNA
2: λDNA-*Hin*d III Digest marker
3: 5.3-kb fragment of pSLJ 4D4

Fig 3.1B Analysis of miniprep recombinant plasmid pSLJ 58210 by 1% agarose gel electrophoresis.
Lane 1: λDNA-*Hin*d III Digest marker
2: pSLJ 4D4 control
3: recombinant plasmid from transformant no. 1
4: recombinant plasmid from transformant no. 2

By performing *Bam*H I and *Nco* I digestion on the recombinant plasmid DNA, a fragment with a size identical to the TCS cDNA cleaved from the pET 58210 was found (Fig. 3.1C). No similar fragment was cleaved from the pSLJ 4D4 DNA.

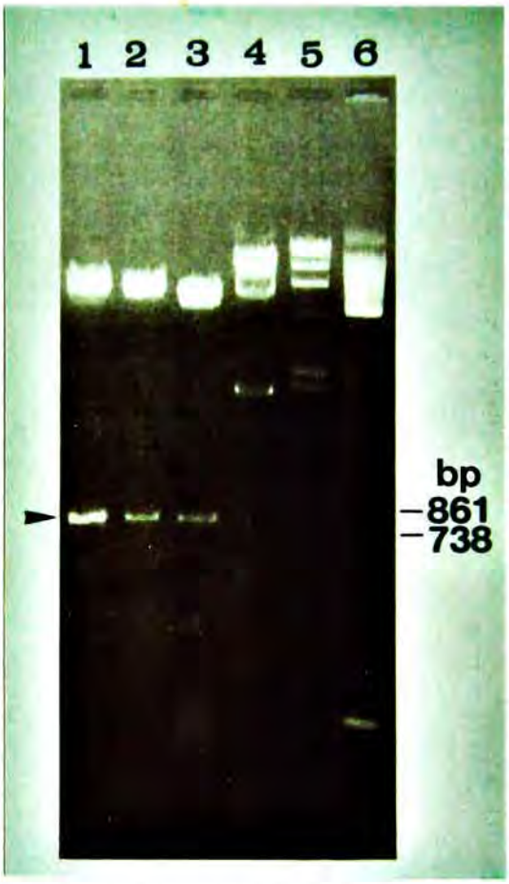


Fig. 3.1C Analysis of *Bam*H I & *Nco* I digestion products of recombinant plasmids pSLJ 58210 and pSLJ 4D4 control by 1% agarose gel electrophoresis.

Lane 1: recombinant plasmid from transformant no. 2 digested with *Bam*H I & *Nco* I
2: recombinant plasmid from transformant no. 1 digested with *Bam*H I & *Nco* I
3: pET 58210 digested with *Bam*H I & *Nco* I
4: pSLJ 4D4 digested with *Bam*H I & *Nco* I
5: λDNA-*Hin*d III Digest marker
6: 123-bp ladder marker
Arrow shows the 0.75-kb fragment containing the TCS cDNA

3.2.2. Construction of the recombinant binary vectors pSLJ TCS 1 and pSLJ TCS 2

The strategy of construction is shown in Fig. 2.3 and methods are described in Section 2.3.2. In the process, pSLJ 58210 DNA was digested with restriction enzymes *Bgl* II and *Hin*d III while the binary vector pSLJ 44024 and pSLJ 44026 were separately digested with restriction enzymes *Bam*H I and *Hin*d III. After digestion, all of the digestion products were analyzed by 1% agarose gel electrophoresis in TAE buffer and the results were shown in Fig. 3.2A. After electrophoresis, the 1.75-kb fragment, carrying the chimeric construct 5′35S-TCS-ocs3′, cut from pSLJ 58210 was purified by GeneClean® kit. The 26-kb fragments of pSLJ 44026 and pSLJ 44026 were purified by Phenol/Chloroform extraction. Afterwards, the 1.75-kb fragment of the chimeric construct 5′35S-TCS-ocs3′ was ligated to the 26-kb fragments of pSLJ 44024 and pSLJ 44026 separately to create the binary constructs pSLJ TCS1 and pSLJ TCS2.

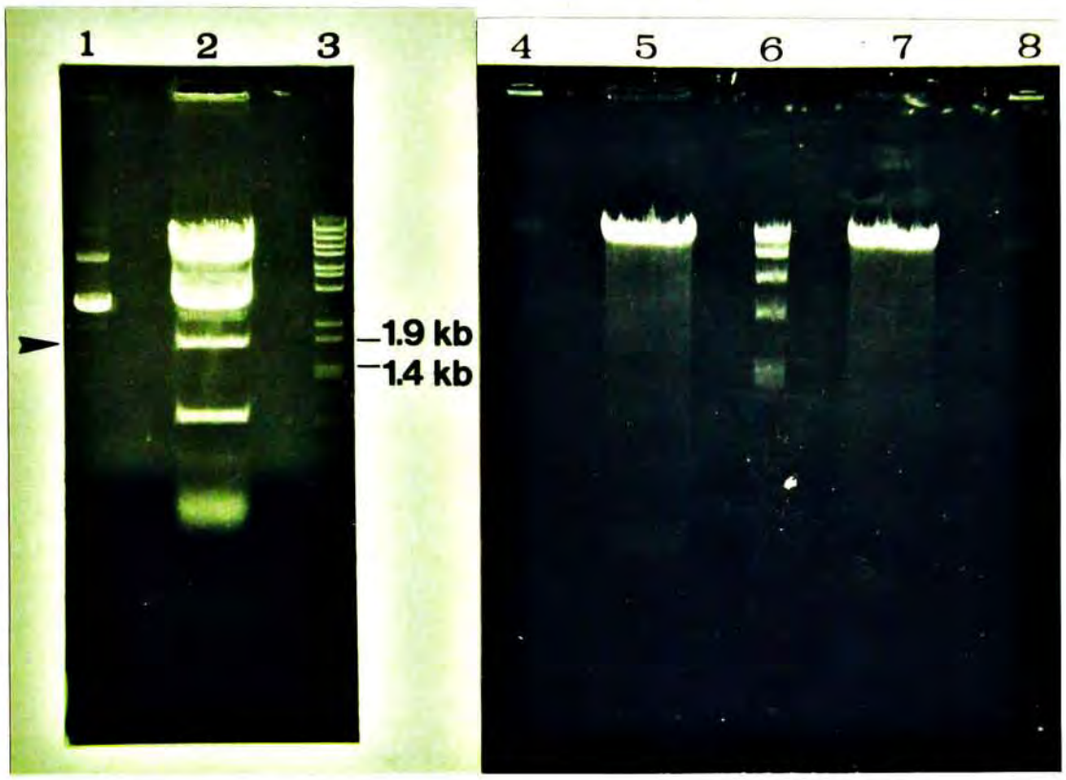


Fig. 3.2A Analysis of restriction digestion products of pSLJ 58210, pSLJ 44024 & pSLJ 44026 by 1% agarose gel electrophoresis in TAE buffer.

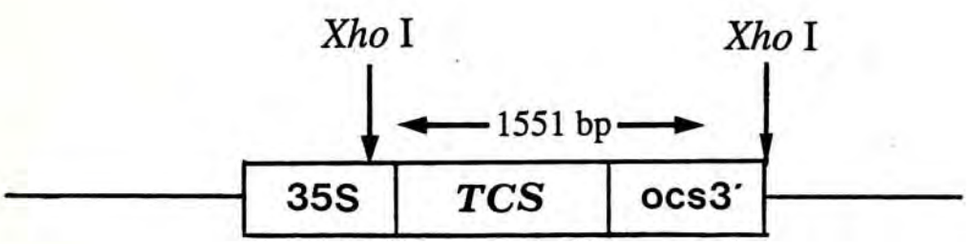
Lane 1: pSLJ 58210 undigested control
2: pSLJ 58210 digested with *Bgl* II & *Hin*d III
3: λDNA-*Bst*E II Digest marker
4: pSLJ 44026 undigested control
5: pSLJ 44026 digested with *Bam*H I & *Hin*d III
6: λDNA-*Hin*d III digest Marker
7: pSLJ 44024 digested with *Bam*H I & *Hin*d III
8: pSLJ 44024 undigested control
Arrow shows the 1.75-kb fragment containing the chimeric TCS cDNA

The ligated DNA was then transformed into *E.coli* strain DH5α. The results of transformation were given in the following table:

Bacteria	Number of transformants
50 μl DH5α (pSLJ TCS2) cell plated	2
100 μl DH5α (pSLJ TCS1) cell plated	50

Screening of transformed colonies

The transformed colonies were screened by minipreparation and *Xho* I digestion. Since the ligation between the *Bgl* II and *Bam*H I site resulted in the disappearance of the recombinant site, two *Xho* I sites around the chimeric TCS cDNA (shown below) were selected in screening.



By performing *Xho* I digestion, fragment with size of about 1.5-kb was formed from the two transformants of DH5α (pSLJ TCS 2) (Fig. 3.2.B). Similar fragment was not found in the pSLJ 44026 digested control. Similarly, 20 out of 50 transformants of DH5α (pSLJ TCS1) were

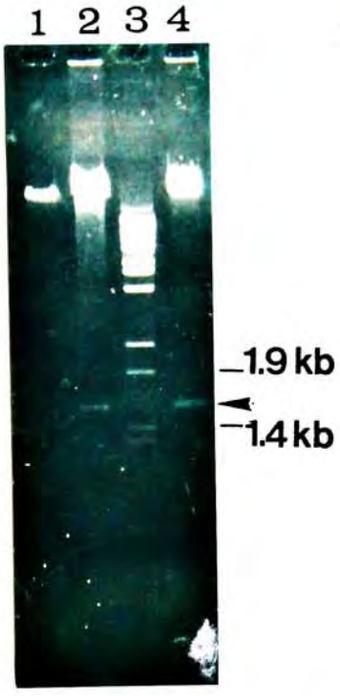


Fig. 3.2B Analysis of *Xho* I digestion products of miniprep plasmid of *E.coli* DH5α (pSLJ TCS2) transformants no. 1and 2 by 1 % agarose gel electrophoresis.

Lane 1: pSLJ 44026 digested control
2: miniprep plasmid of transformant no. 1 digested with *Xho* I
3: λDNA-*Bst*E II Digest marker
4: miniprep plasmid of transformant no. 2 digested with *Xho* I
Arrow shows the 1.5-kb fragment containing the chimeric TCS cDNA

screened firstly by the size of their plasmid DNA using pSLJ TCS2 as a control. Transformant no. 7, 8 and 18 were found to contain plasmid with size similar to the pSLJ TCS2 control while the others had a slightly reduced size (Fig. 3.2C). By performing *Xho* I digestion on the plasmid DNA of transformants no. 7, 8, 18 and also on the pSLJ TCS2 DNA as the digested control, a fragment with the same size as the pSLJ TCS2 digested control was cleaved from the plasmid of transformant no. 18 (Fig 3.2D). There was no similar fragment in the pSLJ 44024 digested control.

3.2.3. Conjugation of pSLJ TCS1 and pSLJ TCS2 into *Agrobacterium tumefaciens* via Triparental Mating

Triparental Mating

Seven combination of bacterial mixtures were prepared (Section 2.3.3) and allowed to mate via conjugation on LB plate. After 24 hours of mating, a 'loopful' of cells was removed from each plate and streaked for single colonies on selective medium (no. 1–7). Results showed that bacterial colonies could only grow on plates no. 6 and 7 after the first streaking. However, colonies could only grow on plate no. 7 after

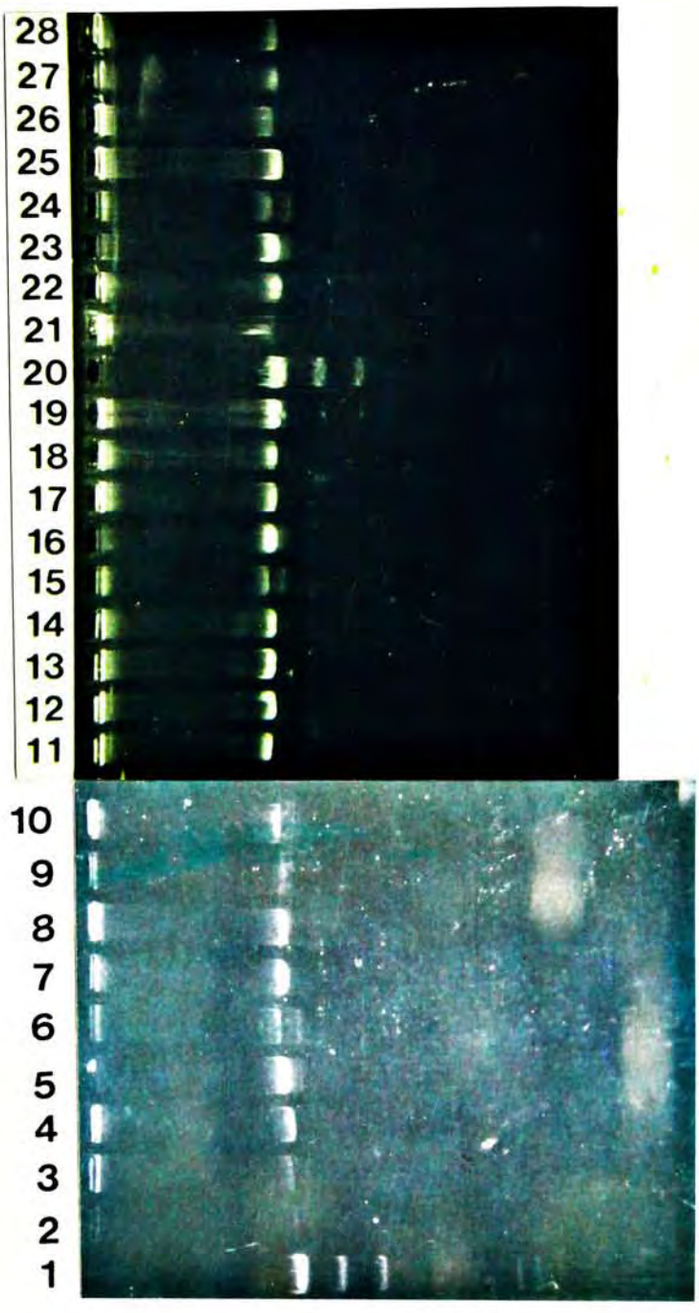


Fig. 3.2C Analysis of the size of miniprep plasmid DNA from transformants no. 1–20 by 0.7% agarose gel electrophoresis.

Lane 1 & 20 : λDNA-*Hin*d III Digest marker
2 – 4 : transformants no. 1– 3
5, 16 & 23 : pSLJ 44024 control
6, 15 & 24 : pSLJ TCS2 control
7 – 14 : transformants no. 4 – 11
17 – 19 : transformants no. 12 – 14
21 & 22 : transformants no. 15 & 16
25 – 28 : transformants no. 17 – 20

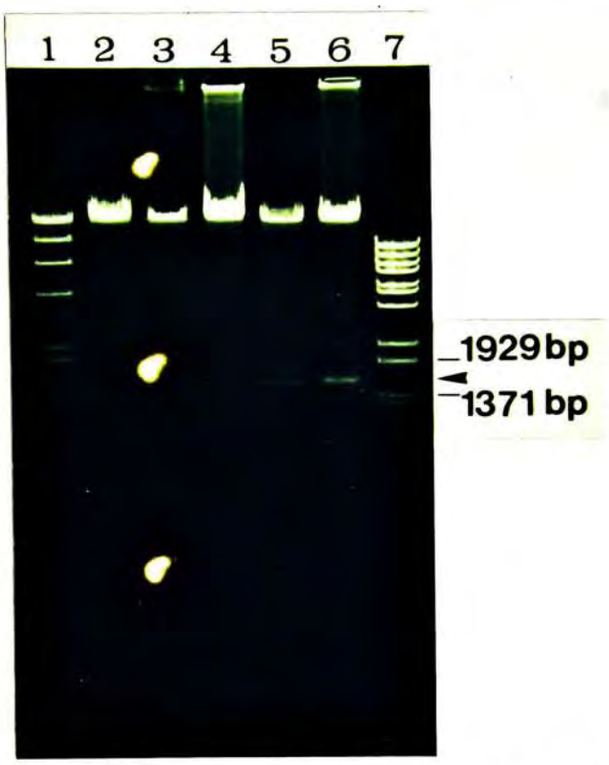


Fig. 3.2D Analysis of *Xho* I digestion products of miniprep plasmid of *E.coli* DH5α (pSLJ TCS1) transformants no. 7, 8 & 18, by 1% agarose gel electrophoresis.

Lane 1: λDNA-*Hin*d III Digest marker
2: pSLJ 44024 digested control
3 & 4: transformants no. 7 & 8
5: pSLJ TCS2 digested control
6: transformant no. 18
7: λDNA-*Bst*E II Digest marker
Arrow shows the 1.5-kb fragment containing the chimeric TCS cDNA

restreaking. This indicated that only *A.tumefaciens* carrying the plasmid pSLJ TCS1 can survive on the selection medium.

Minipreparation of plasmid DNA from transconjugants

The binary plasmids pSLJ TCS1 and pSLJ TCS2 were isolated from the transconjugants by the Magic™ Minipreps (Section 2.2.9) and analyzed by 1% agarose gel electrophoresis (Fig. 3.3A). Results showed that plasmids DNA of size similar to the pSLJ TCS1 DNA control were isolated from the transconjugants. No similar plasmid DNA was isolated from the native LBA 4404 control.

Restriction enzyme digestion of plasmids from transconjugants

By performing *Xho* I digestion, fragments with size similar to the TCS fragment cleaved from the pSLJ TCS2 control were excised from the transconjugants LBA 4404 (pSLJ TCS1) and LBA 4404 (pSLJ TCS2) (Fig. 3.3B). No such fragment was cleaved from the native LBA 4404 control.

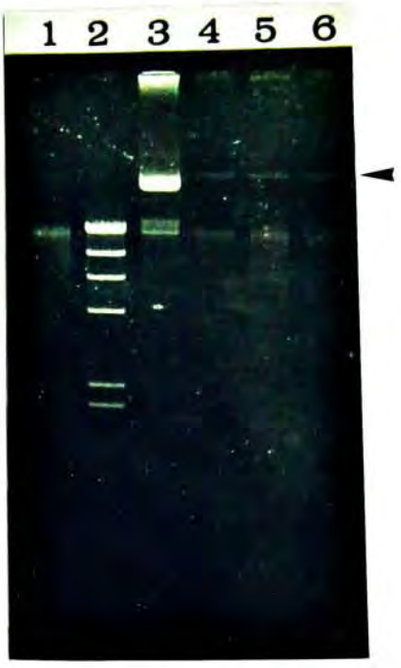


Fig. 3.3A Analysis of Magic™ miniprep pSLJ TCS1 from transconjugant no. 2 and pSLJ TCS2 from transconjugants no. 12 & 23 by 1 % agarose gel electrophoresis.

Lane 1: native LBA 4404 control
2: λDNA-*Hin*d III Digest marker
3: pSLJ TCS1 control
4: binary plasmid from transconjugant LBA 4404 (pSLJ TCS2) no.1
5: binary plasmid from transconjugant LBA 4404 (pSLJ TCS1) no.12
6: binary plasmid from transconjugant LBA 4404 (pSLJ TCS1) no.23

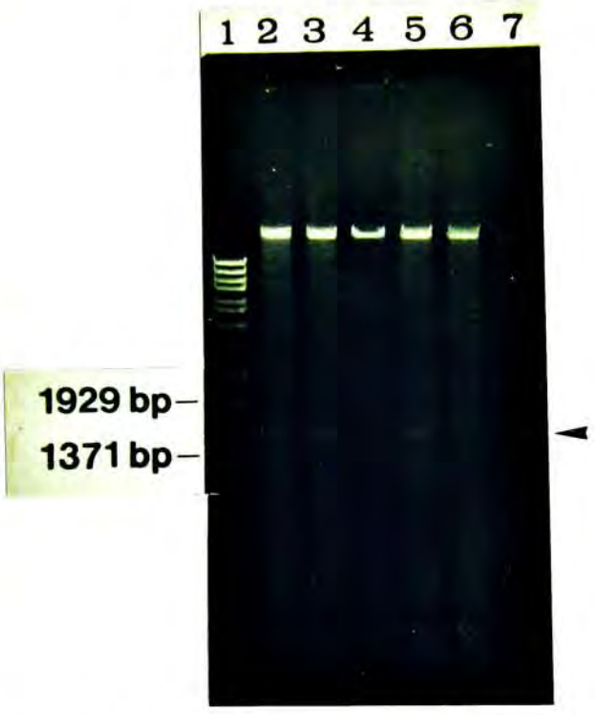


Fig. 3.3B Analysis of *Xho* I digestion products of plasmid from transconjugants LBA4404 (pSLJ TCS1) and LBA 4404 (pSLJ TCS2) by 1% agarose gel electrophoresis.

Lane 1: λDNA-*Bst*E II Digest marker
2 & 3: transconjugants LBA4404 (pSLJ TCS1) no.12 & 23
4: pSLJ TCS2 digested control
5 & 6: transconjugants LBA4404 (pSLJ TCS2) no.1 & 4
7: native LBA4404 digested control
Arrow shows the 1.5-kb fragment containing the chimeric TCS cDNA

Polymerase Chain Reaction

The succeed of transferring the recombinant binary vectors to LBA 4404 was also confirmed by PCR. PCR was performed with two convergent primers (primer C and forward deletion primer) complementary to the TCS cDNA, spanning a 472-bp sequence of the transgene (Fig. 3.4). After PCR, half of the PCR product was analyzed by 2 % agarose gel in TBE butter. As expected, the 472-bp fragment with the same size as the TCS fragment amplified from the pET 58210 DNA was obtained from the transconjugants no. 1, 12 and 23 but not from the control without DNA (Fig. 3.5).

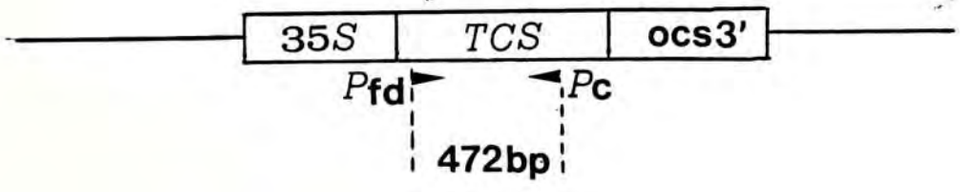


Fig. 3.4 Schematic representation of the chimeric 35S-TCS-ocs3′ transgene. PCR primer (Pfd: forward deletion primer, Pc: Primer C of TCS) are indicated by solid arrows.

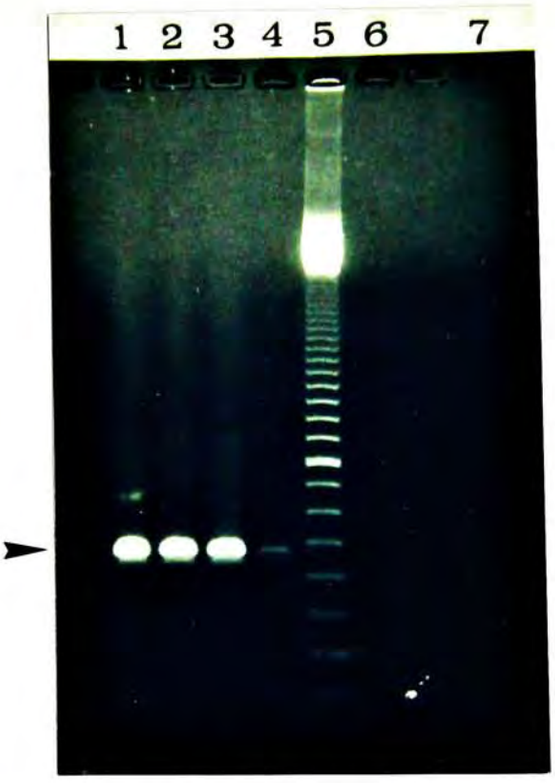


Fig. 3.5 Analysis of PCR product of miniprep plasmid from transconjugants using TCS primer C and forward deletion primer by 1% agarose gel electrophoresis.

Lane 1: transconjugant LBA4404 (pSLJ TCS2) no. 1
2 & 3: transconjugants LBA4404 (pSLJ TCS1) no. 23 & 12
4: PCR product of pET 58210
5: 100-bp ladder marker
6: PCR product of native LBA4404
7: PCR product without template DNA
Arrow shows the 472-bp fragment containing the TCS DNA

3.3. Discussion

Analysis of miniprep recombinant plasmid DNA from the selected transformants by restriction digestion and agarose gel electrophoresis is commonly used to determine whether the required insert has been cloned. Whenever possible, the donor plasmid DNA are also digested to produce the desired insert which can be used as a positive control, since co-migration of small fragments is always more convincing than size comparison of the whole recombinant DNA.

In the construction of pSLJ 58210 DNA, the 0.75-kb fragment of TCS cDNA was ligated to the 5.3-kb fragment of pSLJ 4D4 DNA to create the pSLJ 58210 (Fig. 2.2). Selected transformed colonies were screened by restriction enzyme digestion. Results (Fig. 3.1C) show that desired TCS fragment has been cloned to pSLJ 58210

Therefore in pSLJ 58210, the TCS cDNA is ligated to the CaMV 35S promotor at the 5′ end and to the octopine synthase 3′ polyadenylation sequences at the 3′ end.

In the construction of pSLJ TCS1 and pSLJ TCS2, DNA carrying the chimeric TCS cDNA in pSLJ 58210 was cleaved at the *Hind* III sites 3′ to the polyadenylation sequence, and a *Bgl* II site 200-bp 5′ to the 35S transcriptional start site (Figs. 2.3A & B). Evidence is available that only the 150-bp of the 5′ sequence is needed for full activities of the 35S promoter (Ow *et al.*, 1987). This DNA fragment was then ligated with the *Bam*H I site at the 5′ end and the *Hind* III site at the 3′ end of the multi-cloning site of the binary vector pSLJ 44024 and pSLJ 44026 (Figs. 2.3A & B) and the resulting transformants were screened by restriction digestion and agarose gel electrophoresis. *Xho* I digestion on the pSLJ 44024 and pSLJ 44026 do not produce a 1.5-kb DNA fragment. On the other hand, a fragment of 1.5-kb was found in the successful constructs of pSLJ TCS1 (Fig. 3.2D) and pSLJ TCS2 (Fig. 3.2B).

In the transfer of pSLJ TCS1 and pSLJ TCS2 DNA into *A.tumefaciens* via triparental mating, seven combination of bacterial mating mixtures were set up in LB plate and screened afterwards on agar plates no. 1–7 containing Min T medium supplemented with 50 μg Kanamycin/ml and

500 μg Streptomycin/ml. The *A.tumefaciens* strain LBA 4404 is resistant to streptomycin and is thimine^{+} (able to synthesize thimine). These allow a strong selection for growth on minimal plates after triparental mating, as the *E.coli* donor strains DH5α and HB 101 are thimine^{-}. Both pSLJ TCS1 or pSLJ TCS2 and pRK 2013 DNA carry the neomycin phosphotransferase gene which confers to kanamycin resistance in the transconjugants. Since the pSLJ TCS1 and pSLJ TCS2 carry the wide host range origin of replication, they can replicate in both *E.coli* and *Agrobacterium*. pRK 2013 replicates by ColE1 replicon, and hence cannot be maintained in *Agrobacterium*. Therefore, only the transconjugants *A.tumefaciens* strain LBA4404 (pSLJ TCS1) or LBA4404 (pSLJ TCS2) can survice on the Min T medium containing Kanamycin and Streptomycin in plate no. 7 after triparental mating and plates no. 1–6 are used as controls. Since the typical generation time of *Agrobacterium* is about 2 hours (*E.coli* = 20min), it takes a while for pRK 2013 to be eliminated. So colonies can also grow on plate no. 6 after the first streaking as shown in Section 3.2.3. However, colonies can only grow on plate no. 7 after restreaking.

Agrobacterium cells supposed to contain the pSLJ TCS1 or pSLJ TCS2 DNA were screened. The transfer was confirmed by the appearance of the *Xho* I digested fragments from the transconjugants (Fig. 3.3B).

Polymerase chain reaction (PCR) provides an additional way to confirm the existence of the TCS coding sequence in *A.tumefaciens* LBA4404 (pSLJ TCS1) and LBA4404 (pSLJ TCS2). Since PCR has amplified a specific fragment from transconjugants but not from the native LBA4404 and negative control and these fragments co-migrate with the TCS fragment amplified from the pET 58210 DNA (Fig. 3.5). It is concluded that the transconjugants examined all contain the TCS cDNA.

Chapter 4
Transformation of Tobacco leaf Explants by *Agrobacterium tumefaciens*

Chapter 4 Transformation of Tobacco Leaf Explants by *Agrobacterium tumefaciens*

4.1. Introduction

In order to study the expression of the TCS cDNA in the tobacco plants, *A.tumefaciens* LBA4404 (pSLJ TCS1) constructed (Chapter 3) was used to transform the *Nicotiana tabacum* var. Wisconsin 38 leaf pieces. Although both protoplasts and explants are suitable materials for gene transfer via co-cultivation with *A.tumefaciens*, the use of explants is preferred because of the faster rate of plant regeneration. In the present study, transgenic tobacco plantlets were obtained by direct regeneration of adventitious shoots from transformed leaf pieces in plant culture medium.

Putative transgenic tobacco plantlets were firstly screened by the ability to root in the rooting medium containing kanamycin. The cultured plantlets, which were able to root in the selective medium, were re-established in soil and transplanted to normal growth condition.

After transplantation of cultured transgenic plantlets to normal growth condition, the transgenic tobacco plants expressing TCS were confirmed by the Western blot detection of TCS in the soluble leaf proteins and the level of expression of TCS protein was estimated by the computer software 'ImageQuant'. Those transgenic tobacco plants, with the evidence of TCS expression, were analyzed afterwards for the resistance to TuMV infection.

4.2. Results

4.2.1. Regeneration of leaf explants after transformation

After inoculation of tobacco leaf pieces with *A.tumefaciens* LBA4404 (pSLJ TCS1), the explants were incubated on solid medium for 3 days without antibiotics (Section 2.4.3) for the transformation to occur. The leaf explants were then cultured on shoot induction medium under antibiotic selection (Section 2.4.4). During the period of culture, leaf pieces often swelled up, became twisted and forced the cut edges to lose contact with the medium. Therefore it was required to press the cut edges of the transformed leaf explants back into the medium.

After 5–6 weeks of culture, small shoots regenerated at a frequency of over 90% directly from the cut edge or indirectly from the callus proliferating from the cut edges of the transformed leaf explants (Fig. 4.1A). Regeneration of shoots or growth of callus was not observed in the culture of untransformed leaf explants of wild tobacco species (Fig. 4.1B) under the same selective medium and culture condition. The small shoots were then excised and placed onto the root induction medium (Section 2.4.5). Only 8% of the shoots developed roots after 1–2 weeks of culture. As a result, we obtained 16 transformed plantlets out of 200 leaf explants used for inoculation with *A.tumefaciens* LBA4404 (pSLJ TCS1). Rooted plantlets were removed from the medium and washed with water to remove the agar sticking to the roots (Fig. 4.2). The transformed plantlet was transplanted into soil in a pot which was sealed with clear cling film to keep the moisture (Fig. 4.3). After one week, the film was opened occasionally to allow the plant to acclimatize to the atmospheric environment. The growth of the transgenic tobacco plant at two months after re-establishment in soil was shown in Fig 4.4.

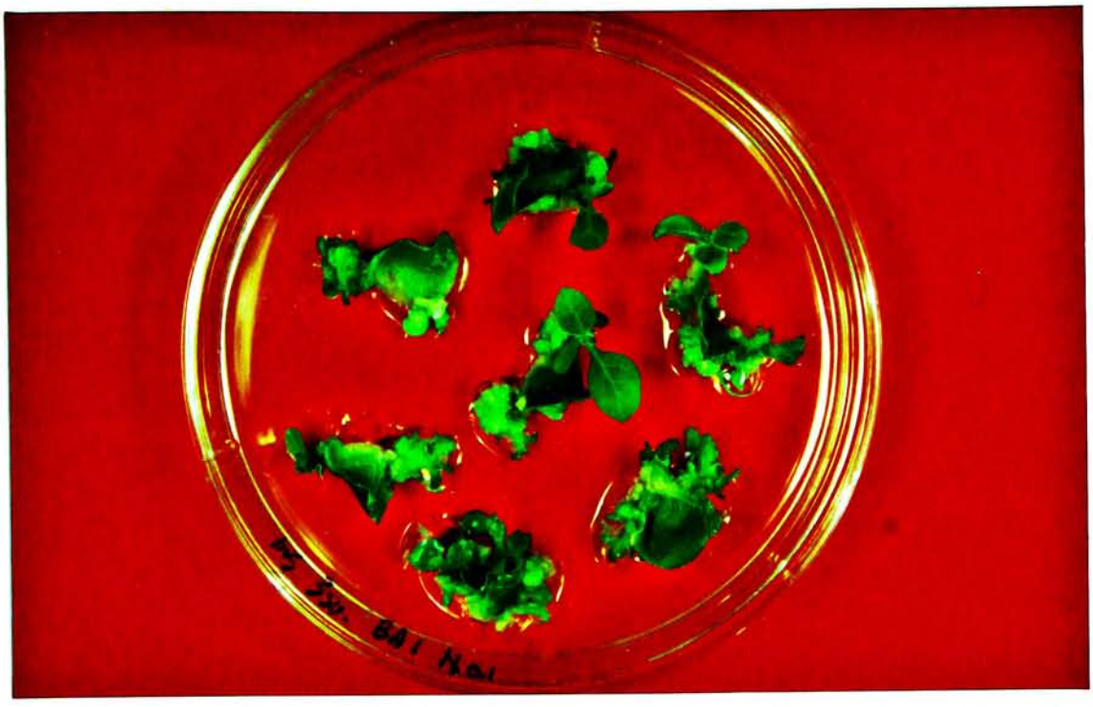


Fig. 4.1A Appearance of leaf explants inoculated with *A.tumefaciens* LBA4404 (pSLJ TCS1) after culture on shoot regeneration medium containing 500 µg Carbenicillin/ml and 100 µg Kanamycin/ml for 5–6 weeks.

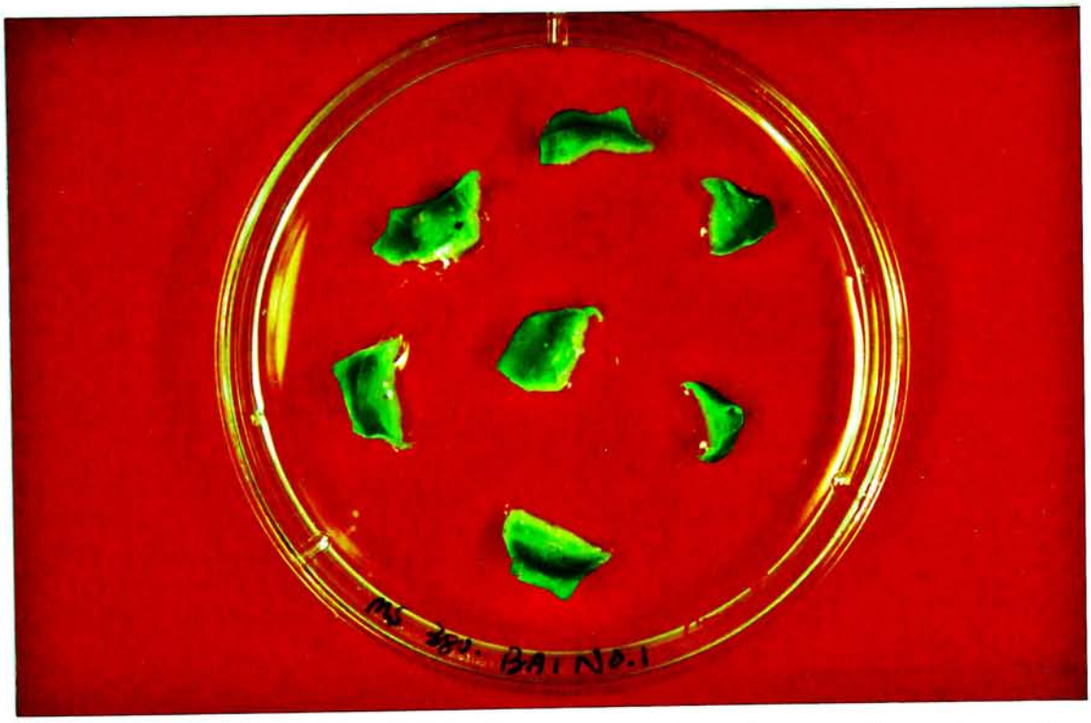


Fig. 4.1B Untransformed tobacco leaf explants cultured on shoot regeneration medium containing 500 µg Carbenicillin/ml and 100 µg Kanamycin/ml for 5–6 weeks.

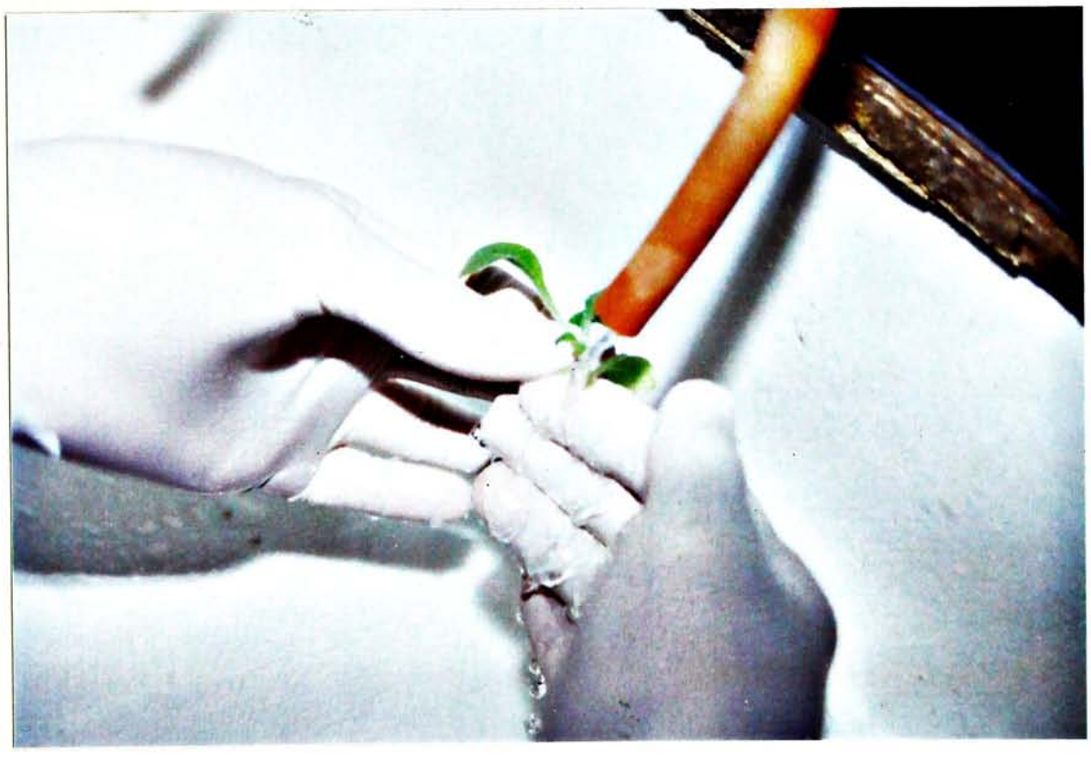


Fig. 4.2 Washing of roots to remove the sticky agar.

Fig. 4.3 Sealing of the pot opening immediately after transplantation.

Fig. 4.4 Appearance of transgenic tobacco plant at two months after transplantation to soil.

4.2.2. The level of expression of TCS in transgenic tobacco leaf

Soluble leaf protein was extracted from the transgenic tobacco and the yield of total soluble protein was determined by the Bradford's method (Section 2.5.1). In the protein assay, a standard curve was created by the BSA solution (Fig. 4.5) and the yield of the 200 μl total leaf proteins extracted from transgenic tobacco samples A, B, C , D and wild type tobacco after correction were about 725 μg/ml, 875 μg/ml, 560 μg/ml, 1063 μg/ml and 3125 μg/ml, respectively.

30 μg of total leaf proteins from transgenic samples A, B, C, D and from the wild species were analyzed by Western blotting (Fig. 4.6). A protein, which was reactive toward anti-TCS serum, was found in the transgenic samples A, B and D but not in sample C and the wild tobacco species. Also, these proteins comigrated with the TCS marker (lane 7). Therefore, the expression of TCS was detected in transgenic samples A, B and D but not in sample C. In Fig. 4.6, the intensity of the bands having the same mobility with the TCS marker, which correlates with the amount of TCS, were analyzed by the computer software 'ImageQuant'. In Fig. 4.7,

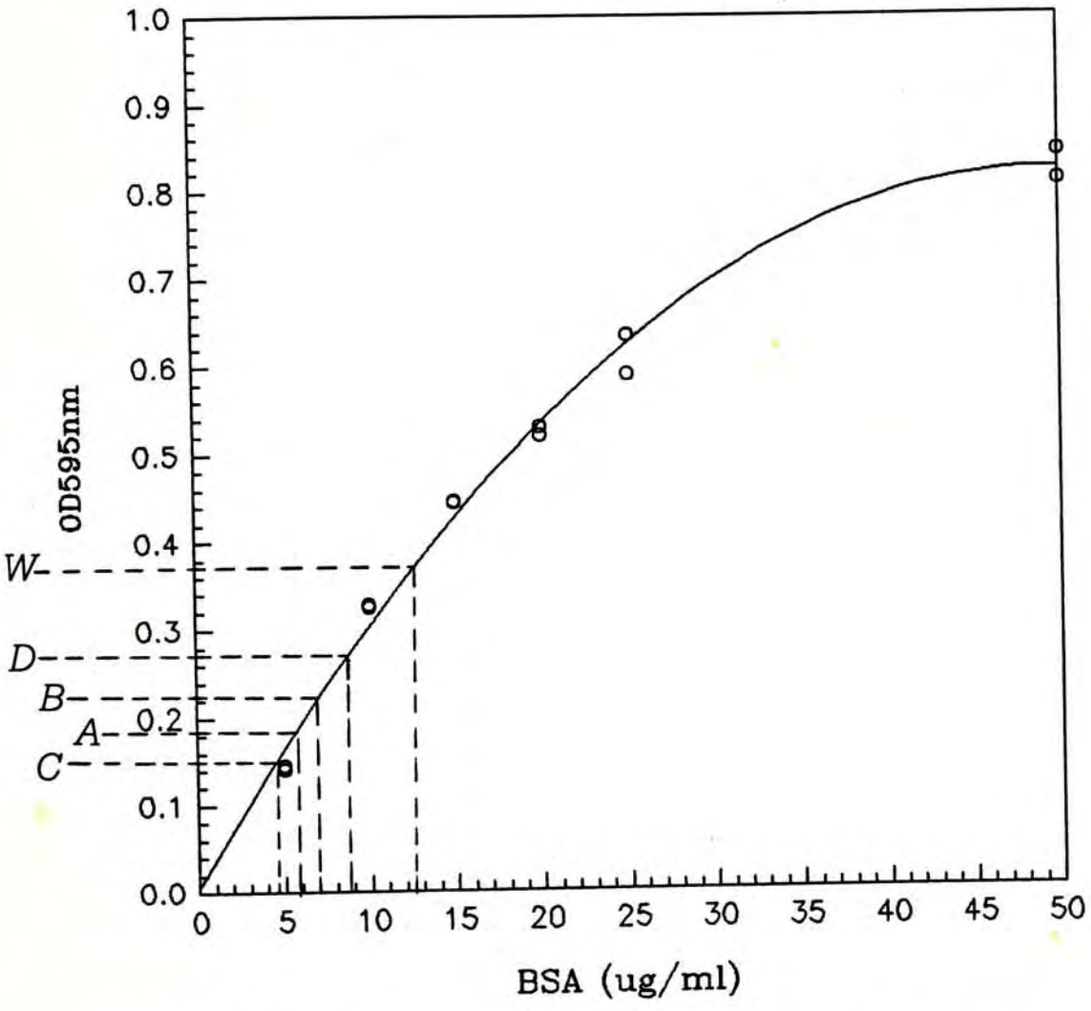


Fig. 4.5 A standard curve of BSA for estimation of the amount of 100-fold diluted soluble leaf protein from transgenic samples A, B, C, D and wild type tobacco (W).

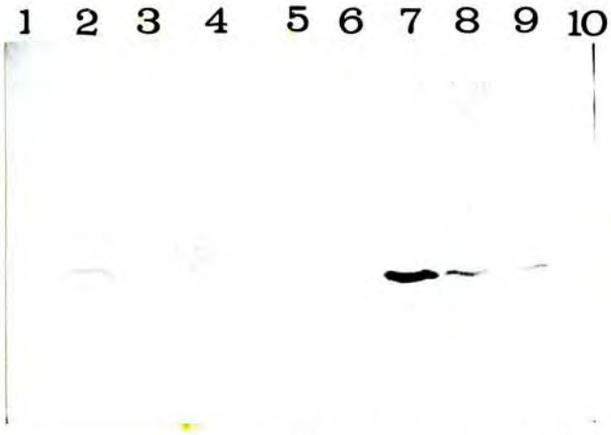


Fig. 4.6. Western blot detection of TCS in transgenic samples A, B, C and D.

Lane: 1. wild tobacco species
2. transgenic sample A
3. transgenic sample B
4. transgenic sample C
5. transgenic sample D
6. Molecular weight marker
7. 500ng TCS marker
8. 100ng TCS marker
9. 50ng TCS marker
10. 10ng TCS marker

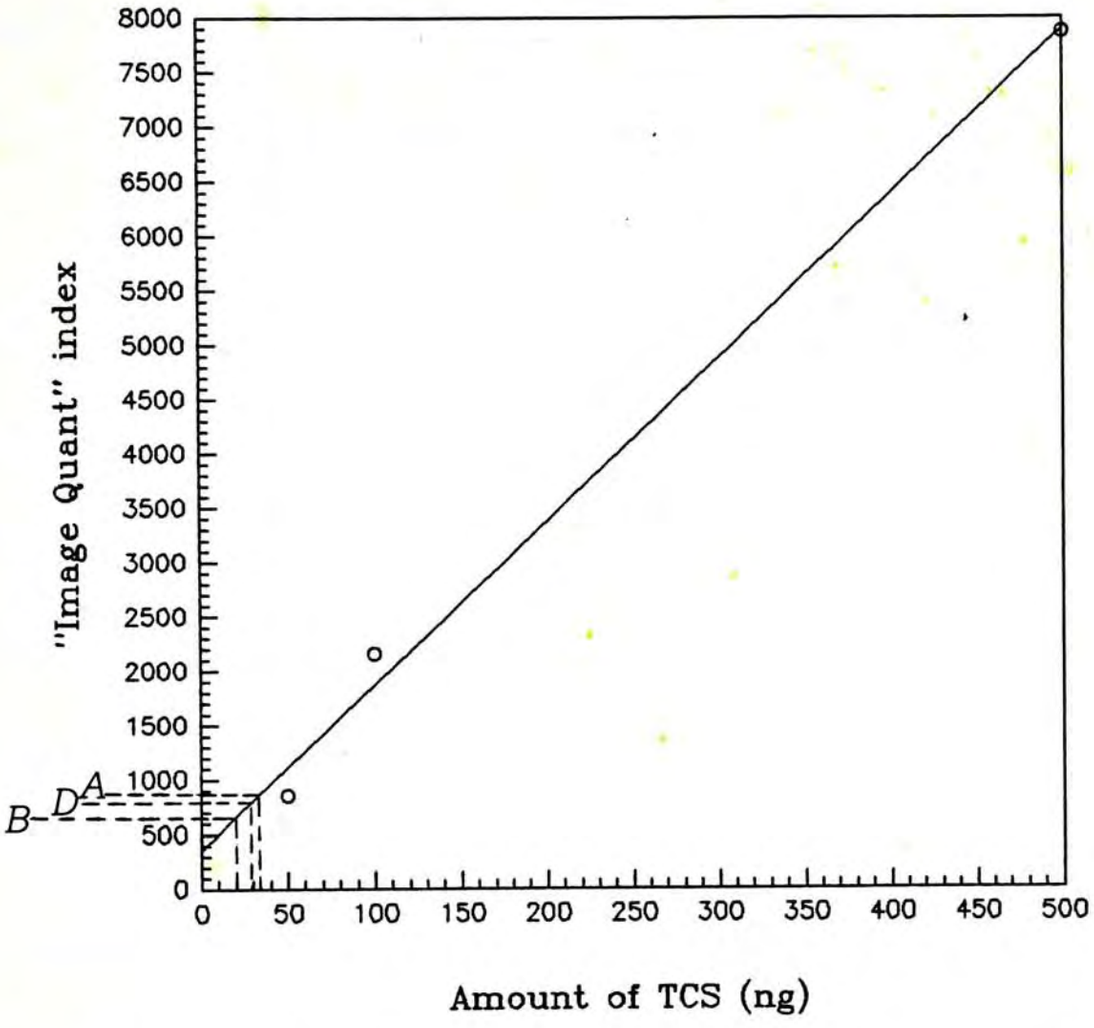


Fig. 4.7 A standard curve of TCS for estimaion of the amount of TCS of transgenic sample A, B & D blotted on the nitrocellulose filter (the amount of TCS protein was about 30 ng in sample A, 20 ng in sample B & 28 ng in sample D per 30 ug of total protein).

the levels of expression, defined as the amount of TCS in the transgenic sample determined from the graph per 30 μg total proteins, times 100, were 0.1% in sample A, 0.06% in Sample B and 0.09% in sample D of the total soluble protein fraction respectively.

4.3. Discussion

4.3.1. Regeneration of transgenic tobacco plants

The leaf disc technique reported by Horsch *et al* (1985) is the most popular method to transform the tobacco plants with *Agrobacterium.* In this method, the explants (leaf disc) are dipped in an overnight culture of *A.tumefaciens* with gentle agitation. The explants are then blotted dry and incubated upside down on solid medium containing a layer of a nurse suspension culture of tobacco cells for 2–3 days to allow transformation to occur. The nurse suspension culture provides various hormones to stimulate plant cell division at cut edges and aid transformation. Based on the method reported by Horsch *et al* (1985), we have performed the transformation experiments except that the nurse suspension culture has not been used. The transformation efficiency was around 8%.

Besides the above classical method, A protocol (De Block *et al.*, 1989) suggested the leaving of the explants in the *Agrobacterium* inoculum for 2–3 days to allow both attachment and transformation. However in our study, many leaf explants degenerated when submerged in the liquid medium for 3 days and bacterial overgrowth could not be controlled even though the explants were washed afterwards with antibiotics. Therefore in our experiments, all the leaf explants were submerged in the *Agrobacterium* inoculum for 10 minutes, and placed on solid medium for 3 days before transferred to the selective medium for regeneration.

After transformation, the leaf explants were selected for growth on the shoot induction medium (Section 2.4.4) containing antibiotics Carbenicillin and Kanamycin. The *Agrobacteria* were removed by the Carbenicillin which was non-toxic to plant cells. Kanamycin is toxic to higher plant cells in which it binds to 70S ribosomes and causes misreading of messenger RNA and thus inhibiting the translation process. However, the transgene encodes the enzyme neomycin phosphotransferase (NPT) which can phosphorylate the kanamycin and therefore prevents its interaction with ribosomes in the plant cells

(Bevan and Flavell, 1983). Consequently, the transformed cells at wound site, having the NPT transgene incorporated in the genome and expressing the NPT transgene, can survive on the selective medium containing kanamycin.

Transformed adventitious shoots along with associated calli can be regenerated from the transformed cells at the inoculated cut edges in the selective shoot regeneration medium containing 1 mg BA/l and 0.1 mg NAA/l (Fig. 4.1A). BA is a kind of cytokinin which promotes shoot formation while NAA is a kind of auxin which initiates root formation. It is found that high cytokinin relative to auxin concentration (10:1) favours shoot formation. From the results, we found that over 90% of inoculated leaf explants can regenerate shoots in the shoot regeneration medium. However, only about 8% of the regenerated shoots form roots in the root induction medium. It may be due to the fact that differentiated structures such as shoots often tend to be naturally less sensitive to the antibiotics kanamycin than roots. Thus, non-transformed shoots are sometimes able to survive the selection process as reported by Horsch et al. (1985) and these are referred to as 'escapes'.

Once the transformed shoot has developed several roots, the plantlet should be transplanted. We found that the agar often sticked to the roots and was difficult to be removed. Since the agar remained on the roots may hinder the respiration of roots in soil and the nutrients in agar may support the undesired microbial growth around the roots, it is advisable to transplant the plantlets to soil when several roots appear at the base of the shoots.

During transplantation, it is necessary to acclimatize the newly regenerated plantlets slowly to the normal growth condition as the fully functional cuticle lacks in the regenerated plant tissue. If the plantlets are transferred directly from a culture vessel to a open environment, the plantlets will often die due to excessive water loss. Hence, the cuticular wax is allowed to build up and the film covering the pot is opened for progressively longer periods. Finally, the acclimatized plantlets can be exposed to the normal growth condition and are allowed to grow to several leaf stage for analysis afterwards.

4.3.2. Expression of TCS in transgenic tobacco plants

Transgenic *N.tabacum* plants (samples A, B and D) accumulated 20–30 ng of TCS per 30 μg (0.06–0.1%) of total protein. Similar study has reported that transfected *Nicotiana benthamiana* plant produced under a RNA viral-based transfection system, accumulated α-TCS, encoded by genomic DNA, to level of at least 2% of total protein (Kumagai *et al.*, 1993). While transgenic *N.tabacum* plants that had lower levels (1–5 ng/mg protein) of PAP were fertile and normal in appearance but those which accumulated above 10 ng/mg protein of PAP tended to have a stunted, mottled phenotype (Lodge *et al.*, 1993) and the plants that accumulated the highest level of PAP were sterile. It seems that the level of expression of TCS in our transgenic tobacco is much higher than that in the transgenic tobacco expressing PAP. In fact, our transgenic tobacco plants (Samples A, B and D) tend to have pale leaves (Fig. 4.8), especially the old leaves are almost bleached. Pale leaves were also be observed but to a relatively less extent in transgenic sample C which does not accumulate detectable level of TCS. The possible reasons for the unhealthy phenotype may be due to the integration of foreign DNA into specific gene sequence which is essential to the chlorophyll

synthetic pathway in plants. However, this suggestion is unlikely since the chlorophyll synthesis seems to be normal in young leaves (Fig. 4.8).

In a similar study of generating PAP preprotein in transgenic tobacco plants, there is a 22 amino acids signal peptide for PAP. Hence, PAP translocates to the cell wall before the signal peptide is cleaved to form the mature protein. The plants thus protect their own ribosomes by compartmentalizing the PAP protein in the cell wall (Ready *et al.*, 1986; Lodge *et al.*, 1993). In our study, the TCS cDNA does not contain the signal sequence and so the expressed TCS should only accumulate in the cytoplasm and exerts itself on the plant ribosomes. Therefore, the unhealthy phenotype may correlate with the expression and the potent ribosome inactivating activity of TCS. The inhibition on the plant ribosomes may be partial since the plant can still survive and grow. The harmful effect of TCS on the old leaves is most obvious since the ribosomes of the old leaves are exposed to TCS at a longer duration. Some transgenic tobacco plants are being grown to observe if TCS would affect their ability to reproduce.

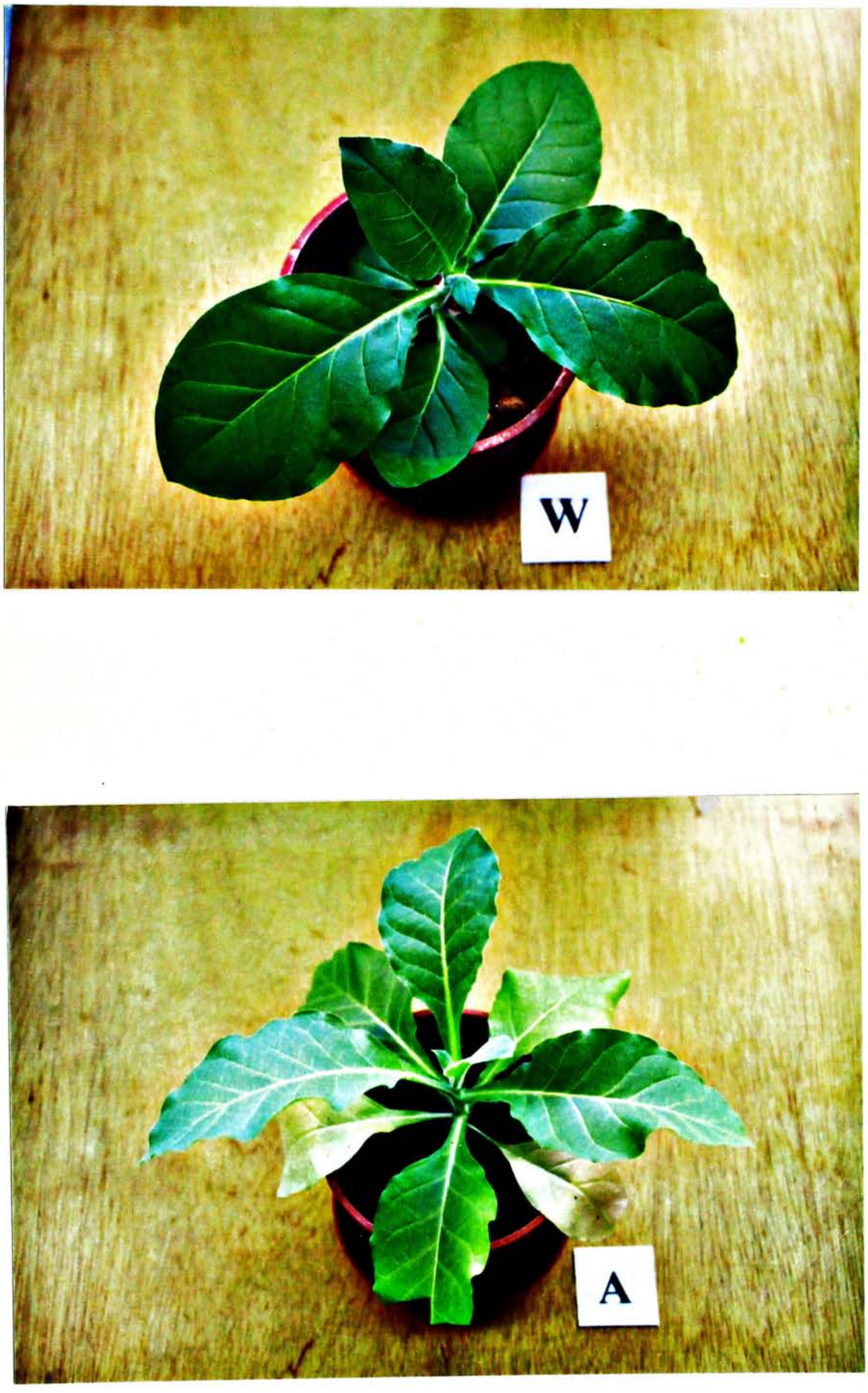
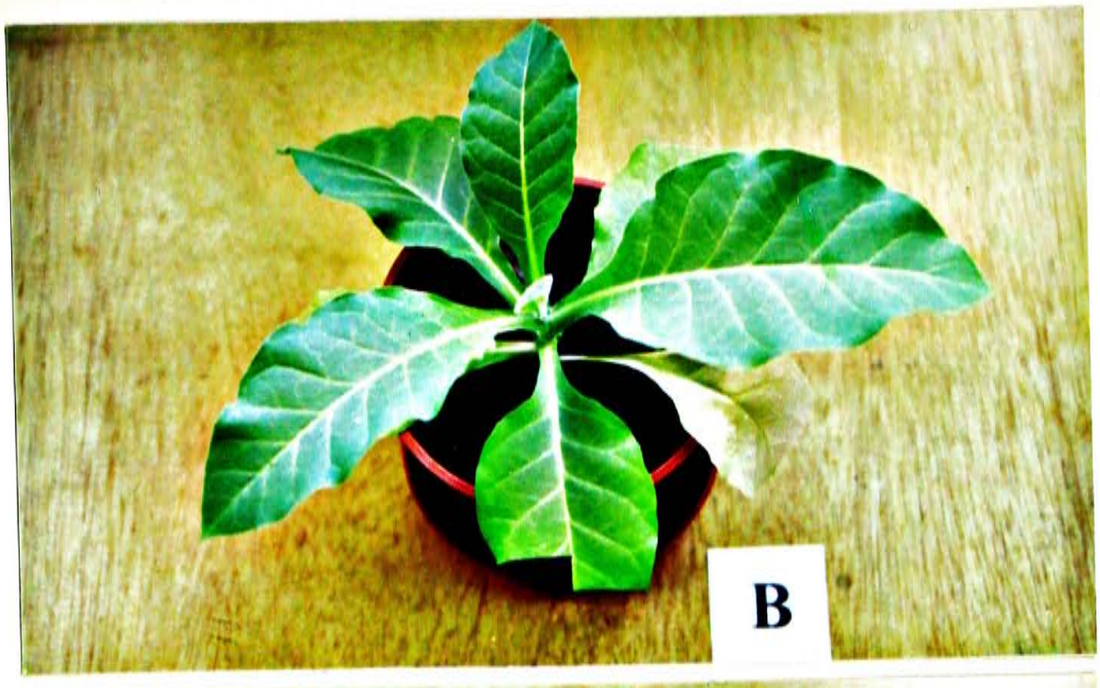


Fig. 4.8 Appearance of transgenic tobacco plants (samples A, B, C and D) and wild type tobacco plant (W).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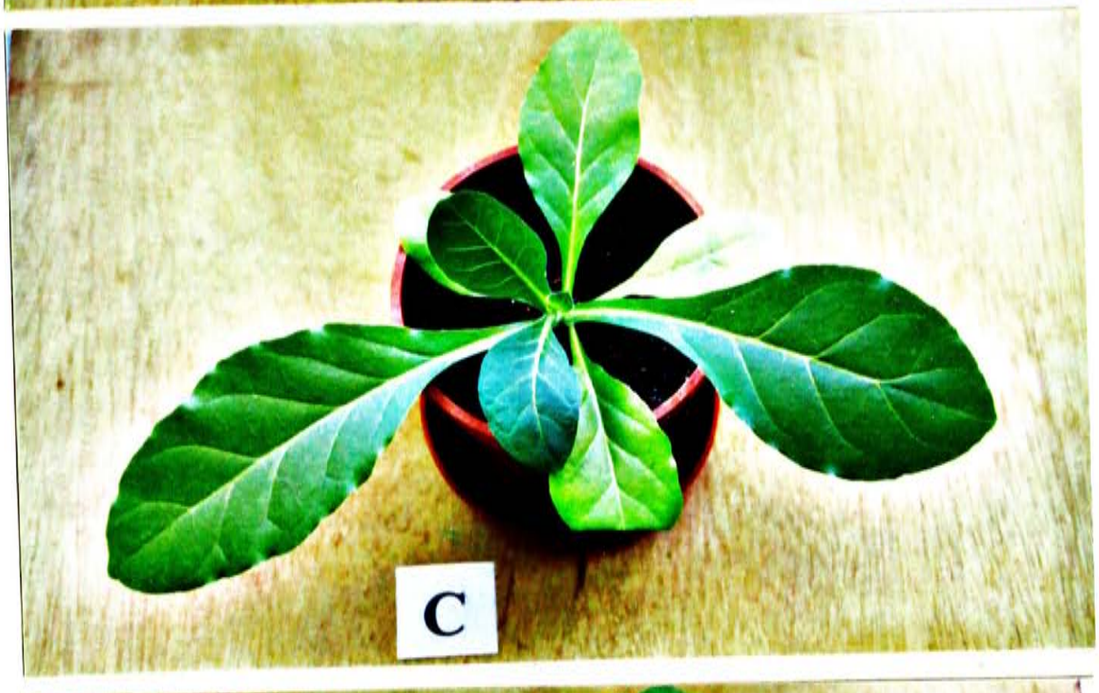
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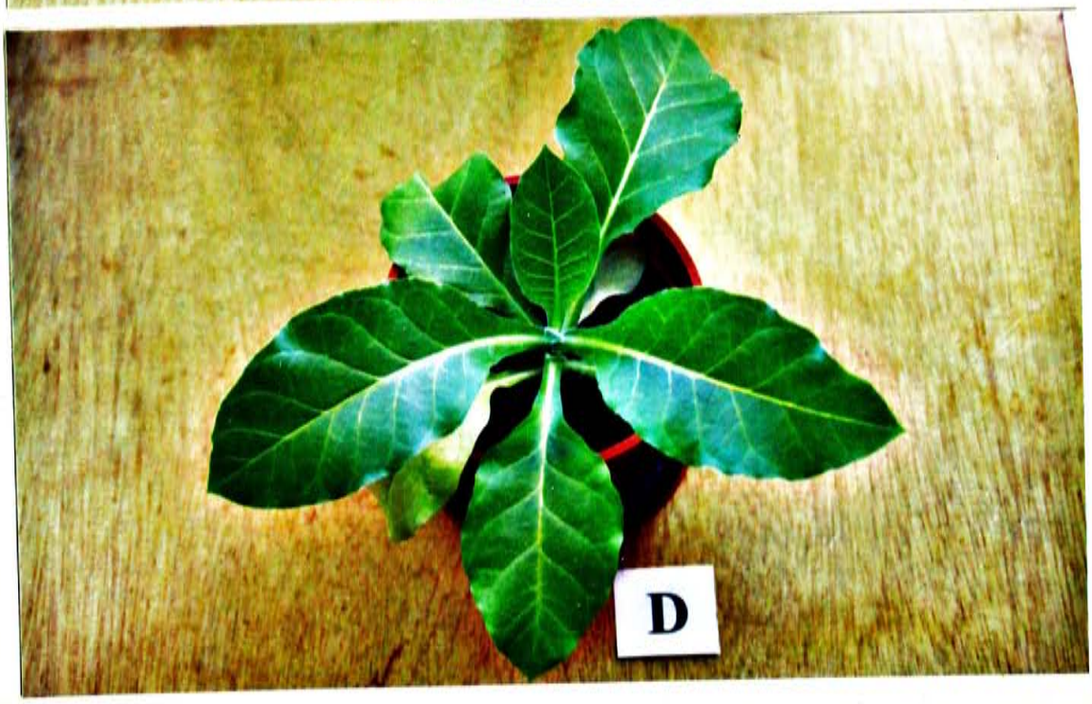

D

Chapter 5
Two approaches to study the Inhibitory effect of TCS on TuMV

Chapter 5 Two approaches to study the Inhibitory effect of TCS on TuMV

5.1. Introduction

Exogenous application of other RIPs such as PAP, Abrin, Ricin, Modeccin, Gelonin, Momordin, MAP (Tomlinson *et al.*, 1974; Stevens *et al.*, 1981; Kubo *et al.*, 1990), respectively has been shown to be effective in preventing these plants from viral attack. Therefore, our first approach to study the inhibitory effect of TCS on plant virus is by applying this protein on two plants *Nicotiana tabacum* and *Brassica parachinensis.* Efforts were made to express and purify the recombinant TCS protein so that sufficient amount of TCS can be obtained for exogenous application. TuMV was propagated in *B.chinensis* and then purified for keeping the virus stock. Then anti-viral assay was performed on a local lesion host (*Nicotiana tabacum* var. Wisconsin 38) and a systemic host (*Brassica parachinensis* var. 80 day) *N.tabacum* is a classical plant model used in the assay of various anti-plant viral proteins. *B.parachinensis* is one of the local food plants which is mostly susceptible to the TuMV infection.

In our second approach, the transgenic *N.tabacum* expressing the TCS gene has been analyzed for the resistance to the challenge of TuMV infection. The results of these experiments described provide a way to access whether TCS is effective to protect the various host plants from the virus infection.

5.2. Results

5.2.1. Expression and purification of recombinant TCS

500 ml *E.coli* strain BL21 (DE3, pLysS pET 58210) which contains a TCS cDNA driven by the T7 DNA polymerase promoter (Zhu et al., 1992) was grown for expression as described in Section 2.6.1. After sonication and equilibration with buffer A, the bacterial lysate was loaded to a CM-Sepharose ion exchange column. The elution profiles of TCS were shown in Figs. 5.1A & B. In Fig. 5.1A, the flow through corresponding to the unadsorbed components of the cell lysate, was washed away by buffer A, while the adsorbed component containing TCS was eluted by the step gradient of 0.5M NaCl. The collected eluate was equilibrated with buffer A overnight and reapplied to the same column, the adsorbed component was eluted by a linear

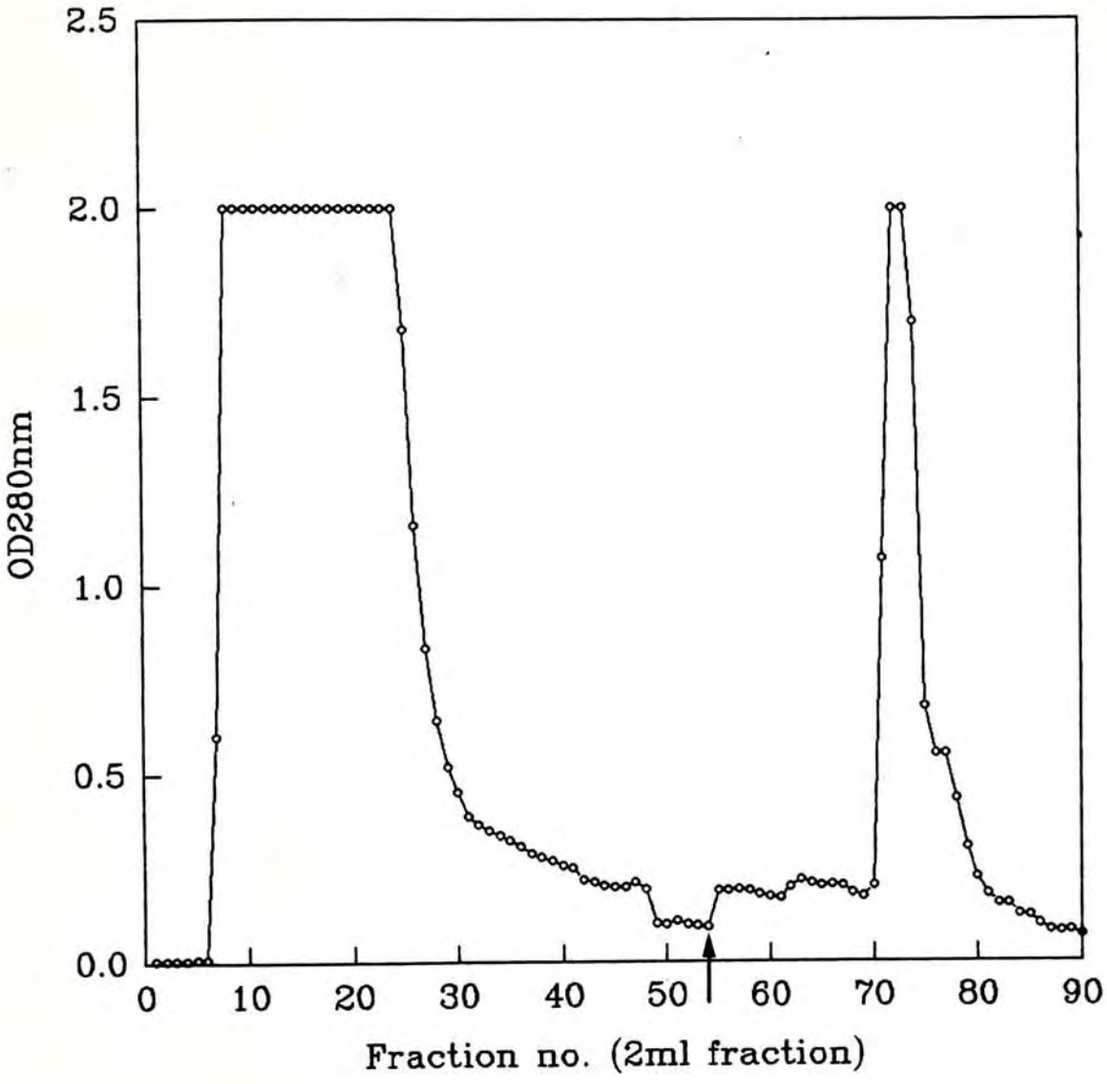


Fig. 5.1A Elution profile of the soluble fraction on CM–Sepharose ion exchange column with step gradient of 0.5M NaCl (Arrow indicates the onset of application of 0.5M NaCl).

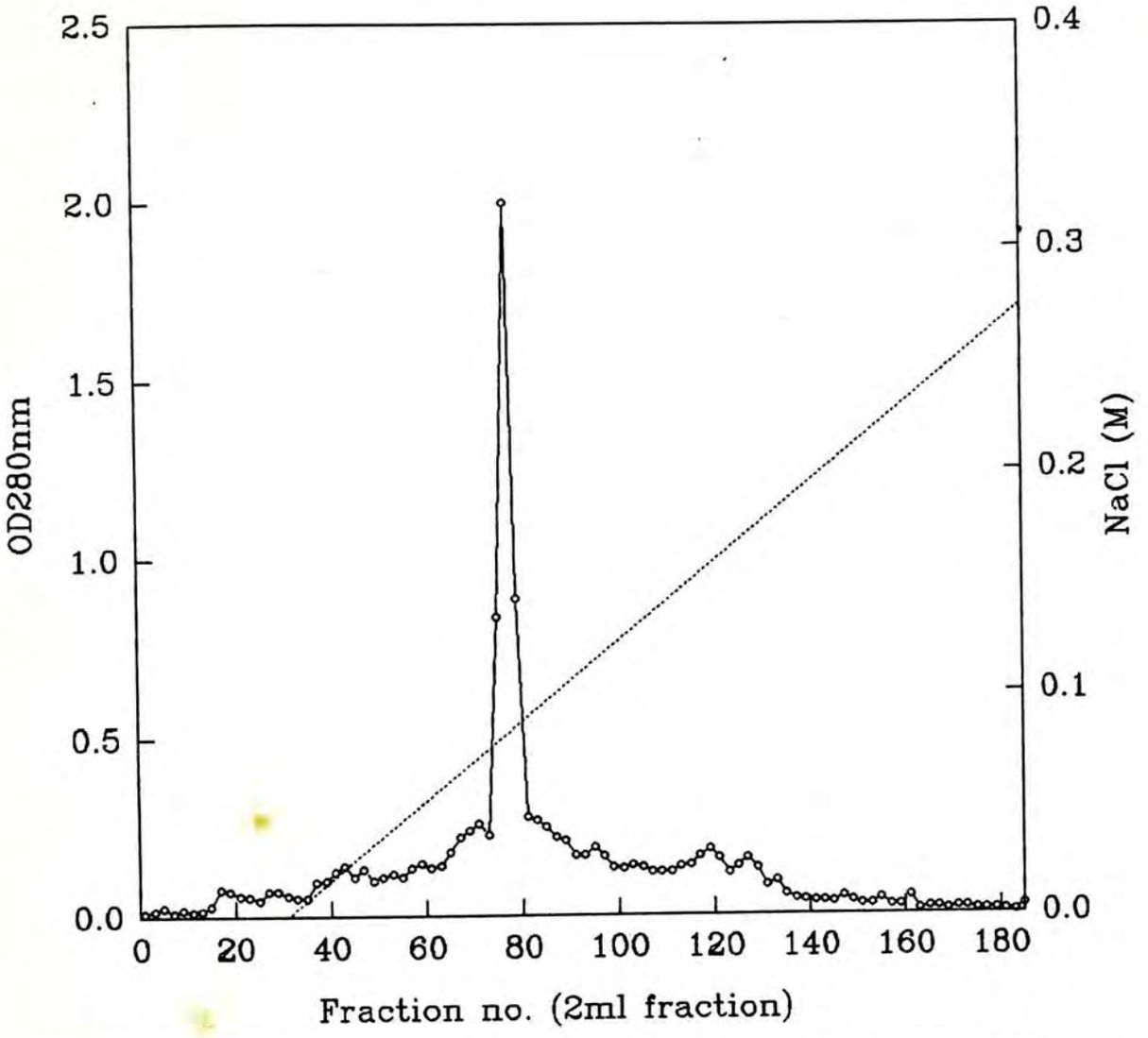


Fig. 5.1B Elution profile of the adsorbed component on CM–Sepharose ion exchange column with linear gradient of 0–0.3M NaCl

gradient of 0–0.3M NaCl and TCS was eluted at about 0.08M NaCl (Fig. 5.1B). Altogether, about 30 mg lyophilized TCS was obtained from 4g of wet bacterial cells in 1 litre culture.

The purity of TCS was analyzed by SDS-polyacrylamide gel electrophoresis. In Fig. 5.2, a single band (Lane 3) was observed which co-migrated with the genuine TCS protein marker (Lane 2). The SDS-PAGE data shown that TCS was in the cell lysate (Lane 8) and was not eluted in the flow through (Lane 7). It was eluted only by the step gradient (Lane 6) or the linear gradient of NaCl (Lane 3).

The identity of TCS was further confirmed by Western blotting (Fig. 5.3) in which the brown bands on the nitrocellulose paper indicated the presence of the TCS and anti-TCS complex.

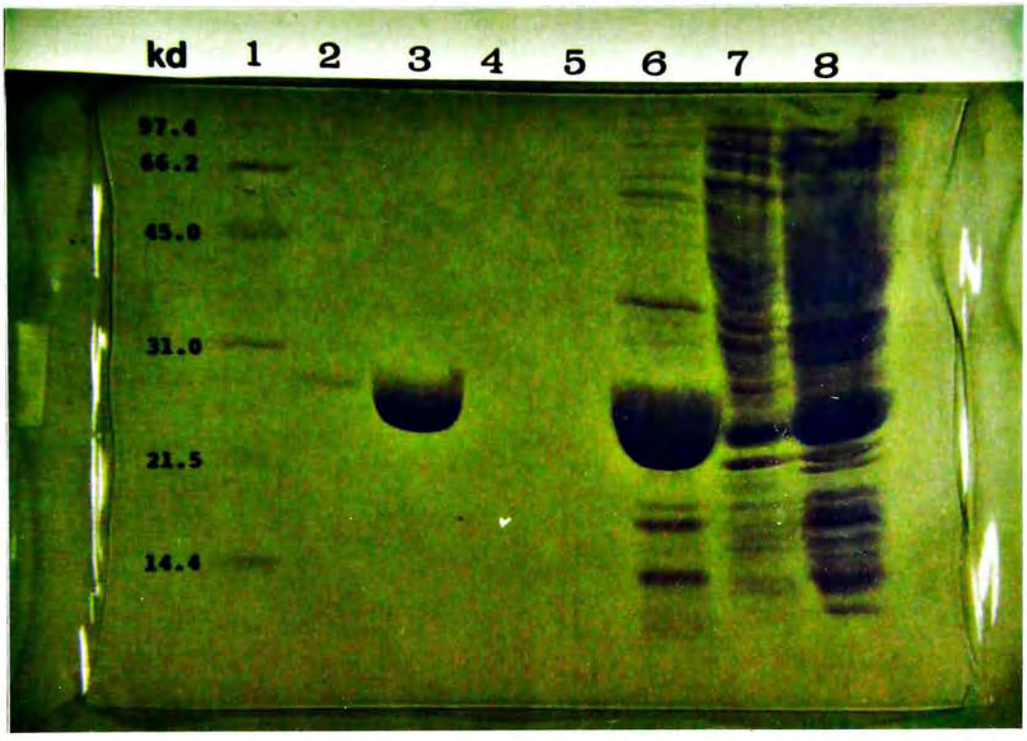


Fig. 5.2 Analysis of different fractions of the CM-Sepharose ion exchange chromatography by SDS-PAGE.

Lane: 1. Molecular weight marker
2. TCS standard
3. Fraction no.77 (adsorbed component) – column 2
4. Fraction no.43 – column 2
5. Fraction no.17 – column 2
6. Fraction no.72 (adsorbed component) – column 1
7. Fraction no.16 (flow through) – column 1
8. Supernatant fraction in cell lysate

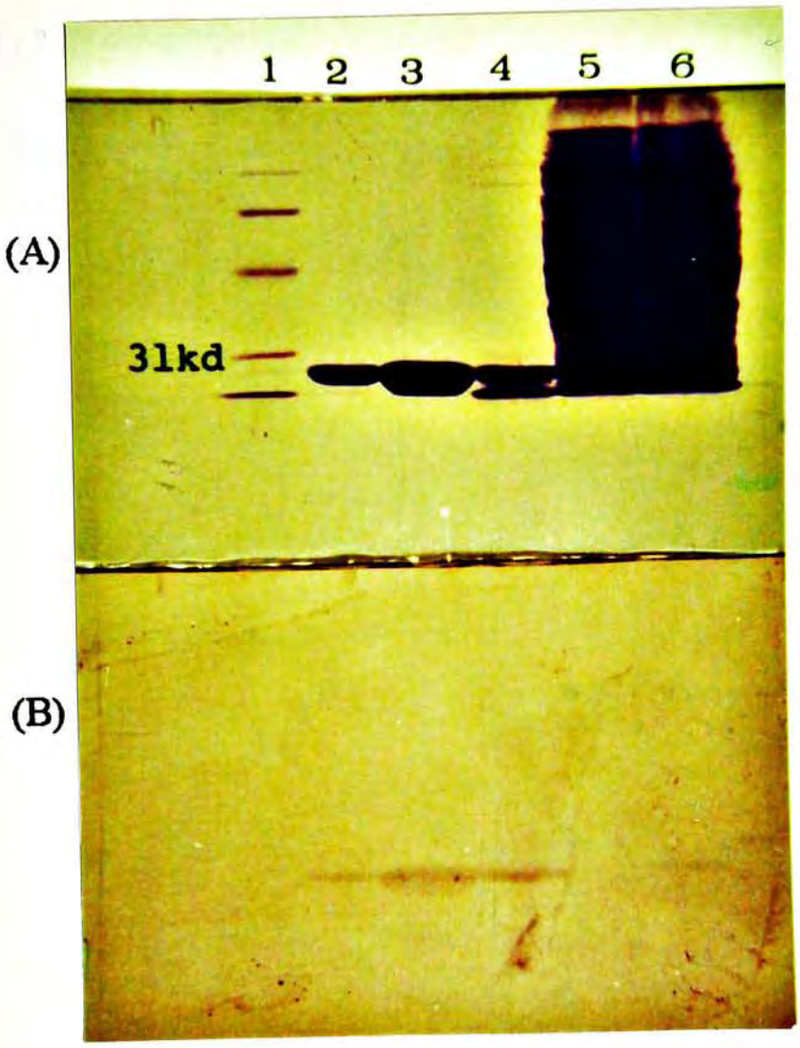


Fig. 5.3 Transfer of proteins from SDS-PAGE to nitrocellulose filter and immunological detection of the immobilized proteins containing rTCS by Western blotting. (A) proteins on SDS-PAGE stained by Coomassie blue. (B) Proteins on nitrocellulose paper stained by immunochemistry method.

Lane: 1. Molecular weight marker
2. TCS standard
3. Adsorbed component (rTCS) – column 2
4. Adsorbed component (rTCS) – column 1
5. Flow through – column 1
6. Supernatant fraction in cell lysate

5.2.2. Purification of TuMV

The TuMV purchased from American Type Culture Collection was propagated in *B.chinensis* as described in Section 2.6.2 and the young leaves expressing mosaic symptom (Fig. 5.4) were harvested after 2 weeks.

In the purification process, the virus was extracted from the infected leaves and was maintained in the extraction buffer containing reducing agent (Na_2SO_3) and chelating agents (Na_2EDTA and DIECA). The virus was isolated from the cell debris and cellular organelles (chloroplast) by differential contrifugation followed by isopynic centrifugation in CsCl. After isopynic centrifugation, a single board band in the centrifuge tube which contained the virus was collected and stored at 4°C. About 80 local lesions were found on tobacco leaf by applying 0.1 ml of a 1:500 dilution sample of the virus.

5.2.3. Anti-viral assay of TCS on local lesion host

Assay of the effect of TCS applied exogenously for acting against TuMV was according to Section 2.6.4. After 7 days of virus inoculation, the

Fig. 5.4 Appearance of mosaic symptoms at young leaves of *B.chinensis* after TuMV inoculation.

expression of local lesions showing virus infection was observed at the control and treated half leaves (Figs. 5.5A & B). The results are summarized in tables 5.1 and 5.2.

Table 5.1. Effect of rTCS on local lesions production by TuMV[a]

Concentration (μg TCS/ml)	No. of local lesion/half leaf		Inhibition (%)
	Control	Treated	
250	56	21	
	8	14	
	8	21	
	30	13	
	34	17	
	10	7	
	19	20	
	87	6	
	43	16	
	8	3	
	Mean[b] 30.3 ± 26.0	13.8 ± 6.5	54
400	16	10	
	54	7	
	87	23	
	165	15	
	123	16	
	34	9	
	74	15	
	16	0	
	29	1	
	81	23	
	Mean[b] 67.9 ± 48.6	11.9 ± 8.0	82

a: 1/500 dilution of purified TuMV

b: Mean no. of local lesion on 10 half leaves of tobacco

Left half: virus control; Right half: TCS (400 μg/ml) treated

Left half: virus control; Right half: TCS (250 μg/ml) treated

Fig. 5.5A. Effect of TCS on local lesion production by TuMV.

Fig. 5.5B Effect of Cytochrome C on local lesion production by TuMV (left half: virus control; right half: 400 μg cytochrome C /ml treated).

Table 5.2. Effect of Cytochrome C on local lesions production by TuMV[a]

Concentration (μg Cyto. C/ml)	No. of local lesion/half leaf		Inhibition (%)
	Control	Treated	
400	36	124	
	17	26	
	44	65	
	34	74	
	48	74	
	75	181	
	77	114	
	29	47	
	77	175	
	27	82	
	Mean[b] 46.4 ± 22.7	96.2 ± 51.6	NIL

a: 1/500 dilution of purified TuMV

b: Mean no. of local lesion on 10 half leaves of tobacco

5.2.4. Anti-viral assay on Systemic host

Assay of the effect of TCS applied exogenously for acting against TuMV was according to Section 2.6.5. After 10 to 13 days of virus inoculation, the expression of mosaic symptom (Fig. 5.4) showing virus infection was observed at the young leaves of all the control plants.The plants treated with TCS were delayed in symptom development compared with the controls. The percentage of plants showing TuMV infection on their young leaves at successive days after virus inoculation was plotted against the day of expression (Fig. 5.6). Difficulties were encountered in

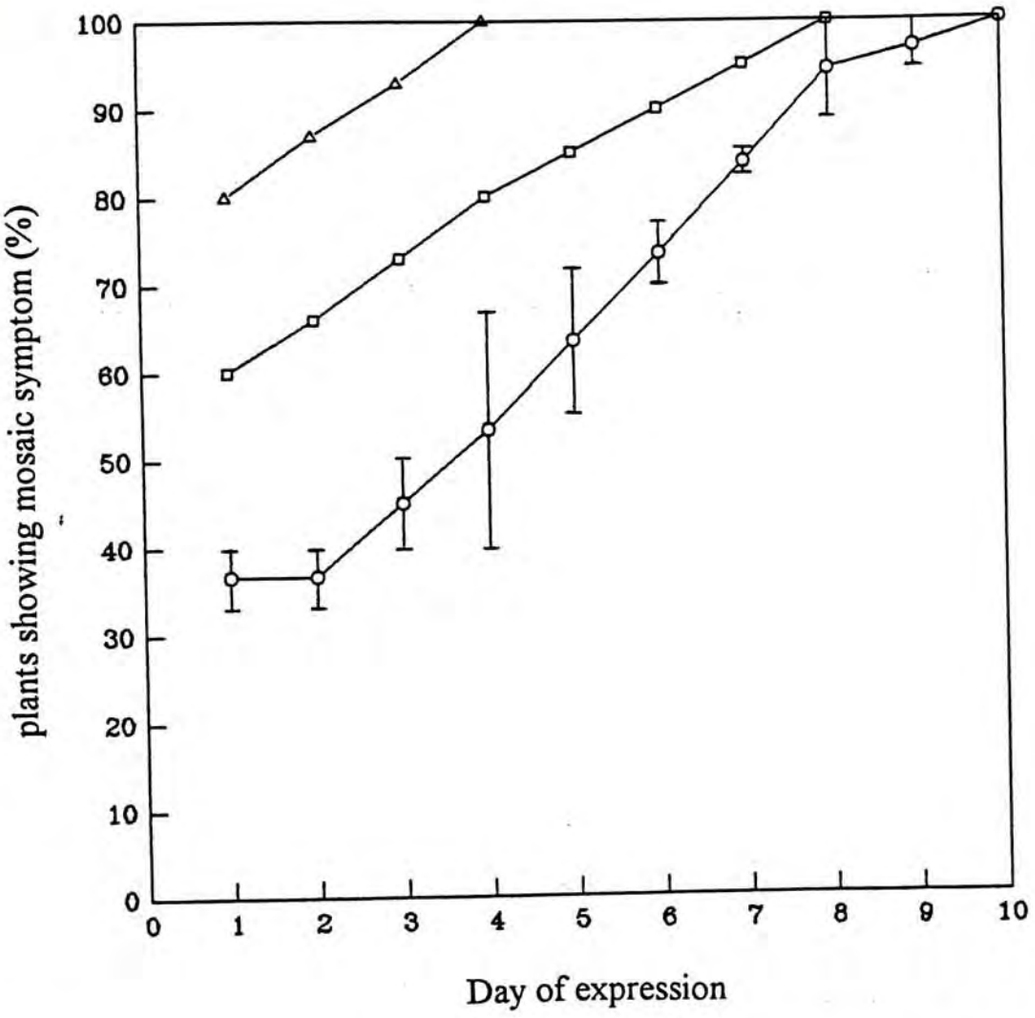


Fig. 5.6 Effect of various concentration of purified TCS on the percentage of plant showing TuMV infection at successive days after virus inoculation.

Δ 1 μg TCS/ml
□ 10 μg TCS/ml
o 100 μg TCS/ml

the experiments as some batches of plants did not show mosaic symptom after virus inoculation. Also poor growth was observed in these batches which may correlate with the unfavourable high temperature (28°C–32°C) at daytime and the acidic soil (pH 4.3–5.4). Poor growth may renders the plants become less susceptible to viral infection. Therefore, the observation for virus symptom in these plants was not included.

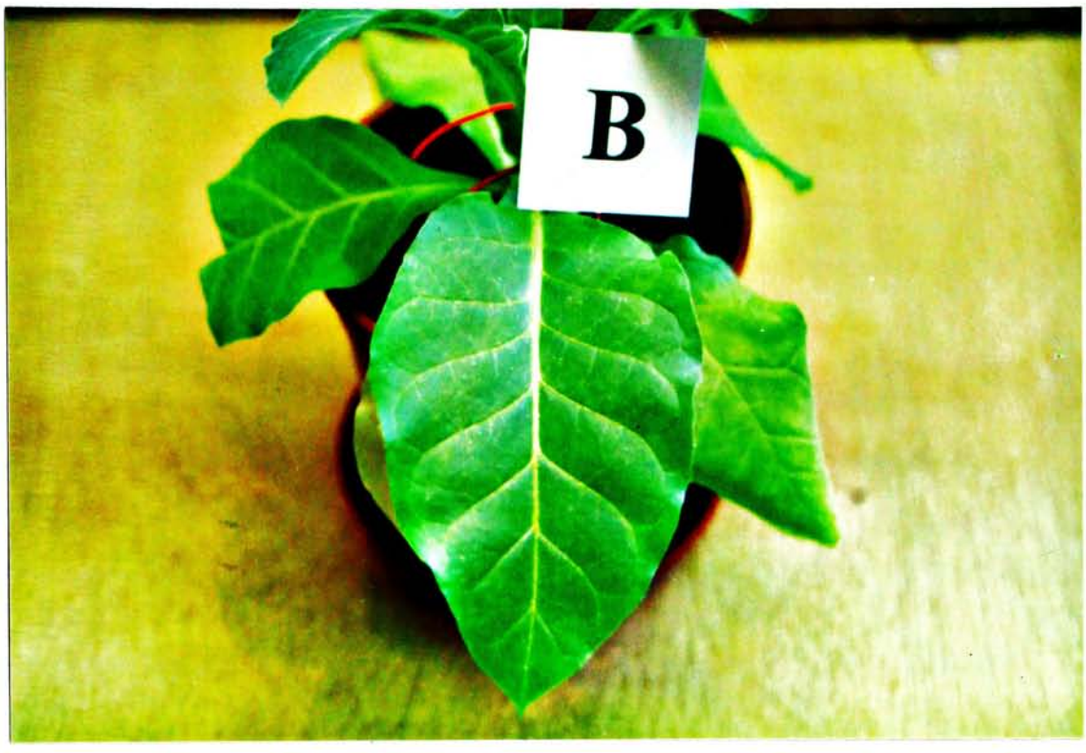
5.2.5. Anti-viral assay of Transgenic tobacco against TuMV

A pair of the opposite leaves in each of the transgenic tobacco samples A, B and D which had been shown to have the expression of TCS protein in the leaves and three wild tobacco species were rubbed with TuMV. After 7 days of virus inoculation, local lesion appeared on the inoculated leaves of the three wild tobacco species, while the transgenic tobacco samples A, B and D did not develop viral symptoms during the observation period of more than 1 month (Fig. 5.7).

(W)

(A)

Fig. 5.7 Appearance of the leaves of the transgenic tobacco samples A (A), B (B) & D (D) and wild type tobacco species (W) after 7 days of inoculation.



(B)

(D)

5.3. Discussion

Recombinant TCS proteins were expressed in *Escherichia coli* and successfully purified to homogeneity. This guarantee that the inhibitory activity on TuMV is due to TCS but not the presence of other possible irrelevant proteins.The TuMV was purified to make a virus stock to be used for subsequent viral assay. In other study, crude extract from the leaves of infected plant species has been used (Kubo *et al.*, 1990). Other proteins or factors in the crude extract may affect the efficacy of infection.

The present study shows that exogenous application of TCS can inhibit the local lesions production by TuMV. From table 5.1, we can see that TCS in 250 μg/ml and 400 μg/ml inhibited the virus lesion by 54% and 82% respectively. It has been reported that 1 mg/ml Mirabilis Antiviral Protein (MAP) inhibited the TuMV lesion in tobacco by 75% (Kubo *et al.*, 1990). A dose response is also observed in our study as in which less local lesions was found as TCS concentration increased. This dose response was also observed in the systemic host *B.parachinensis* in which the delay in the day of mosaic symptom development increased

with the increase in dosage of TCS from 1 to 100 μg/ml (Fig. 5.6). Apparently, incease in the dosage of TCS prolonged the incubation period for the virus or decreased the number of infection sites so that the appearance of systemic symptom was delayed.

We do not yet understand the mechanism of viral resistance contributed by the exogenous application of TCS. One possibility is that TCS, being a RIP, enters the host cells together with TuMV during mechanical inoculation and prevents translation of the viral RNA by inhibiting the protein synthesis of the host cells. Alternatively, TCS may act by binding to the virus and prevent the attachment of the virus to the host cell. Since the possible TCS-TuMV complex formation may be caused by the charge interaction between the basic natured TCS (pI 9.4) and the acidic groups on the virus, cytochrome C (pI 9.6) with pI value similar to TCS was used to find if inhibition of viral infection is caused by the possible complex formation. Table 5.2 shows that the cytochrome C used in the same dosage as TCS did not inhibit the local lesion formation caused by TuMV in host plants. Instead, with cytochrome C, the number of lesions was elevated by 107%. Therefore, it seems that the possibility of

inhibition of viral infection caused by the charge interaction of TCS with the virus is low.

The hypothesis that RIP inhibits viral infection by inactivating the protein synthesis in the infected host cell was also suggested by Prestle *et al.* (1992). They proposed that similar to mammalian ribosomes, a specific adenosine residue in the 25S rRNA of the plant ribosomes is removed by the RIP. Therefore, it appears that TCS may enter the cell along with TuMV during mechanical viral inoculation and inactivates the host ribosomes.

There was 100% of resistance to TuMV infection in three transgenic tobacco plants. This indicates that TCS expressed in the transgenic tobacco plants is responsible for the resistance. In a similar study, transgenic tobacco and potato plants that express PAP shows resistance to infection by three unrelated virus (Lodge *et al.*, 1993). They proposed that during viral infection PAP localized in the cell wall of transgenic plants may bind to the virus or to a component of cell wall and thus preventing virus from entering the cells, or PAP could enter the cell

along with the virus and then inactivates ribosomes to cause inhibition of viral protein synthesis. However in our study, the expressed TCS in transgenic plant does not contain the signal sequence and the protein should only accumulate in the cytoplasm. Therefore, endogenous TCS may bind with the virus after infection or inhibit the viral translation by its ribosome inactivating activity in the host cells. Interestingly, its seems that there is a differential susceptibility between viral and cellular translation. Perhaps plants have some unknown mechanisms, other than by compartmentalizing TCS in the cell wall, to render their own cellular translation become less susceptible to the inhibition by TCS.

In an earlier study, an viral RNA-based vector was used to transfect the *N.benthamiana* with α-TCS consists of the signal sequence. There was no inhibition of replication of the recombinant virions as the transfected plants accumulated recombinant virions similar in size and yield (2.9–8.6 mg/g of fresh weight) to wild type virus in infected tissue (Kumagai *et al.*, 1993). Possible reason may be due to the localization of the α-TCS mature protein in the cell wall and thus isolating α-TCS from ribosomes of the plant cells.

Chapter 6
Establishment of plant culture conditions for efficient shoot regeneration from tissue explants of *B.parachinensis*

Chapter 6 Establishment of plant culture conditions for efficient shoot regeneration from tissue explants of *B.parachinensis*

6.1. Introduction

To investigate which type of explants of *B.parachinensis* is more refractory to the regeneration in culture medium and to optimize the composition of nutrients and growth hormones for efficient shoot regeneration, cotyledon-petiole, hypocotyl segments and the internode stem segments of shoot culture were cultured on nutrient medium supplemented with a range of growth regulators.

6.2. Results

After two weeks of culture, cotyledon petiole showed shoot regeneration (Fig. 6.1) in nutrient medium containing silver nitrate at 5 mg/l and BA at 0.5, 1 and 2 mg/l and the results are summarized in table 6.1. As shown in Fig. 6.2, hypocotyl segments only showed callus proliferation from the explants at all of the four combination of growth regulators but large callus was obtained at condition with 2–6 mg BA/l, 0.5 mg NAA/l and 5 mg silver nitrate/l. Also, after 3–4 weeks of the

Fig. 6.1 Cotyledon petiole explant of *B.parachinensis* showing developing shoots.

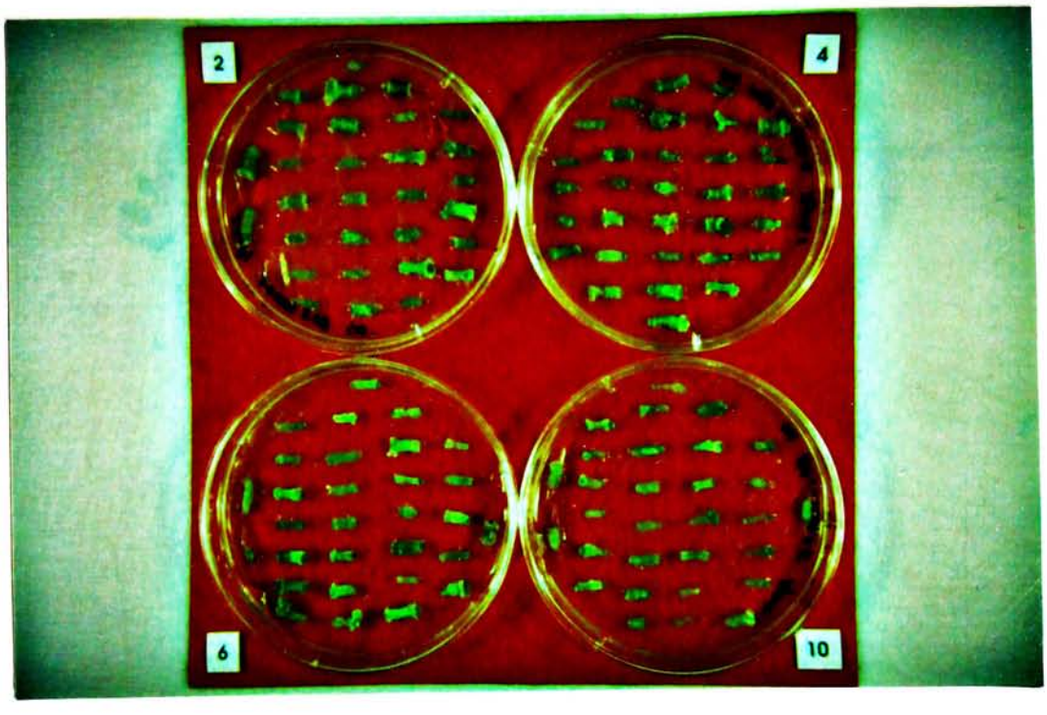


Fig. 6.2 Callus proliferation in hypocotyl explants of *B.parachinensis* at 2, 4, 6 or 10 mg BA/l , 5 mg NAA/l and 5 mg $AgNO_3$/l of culture medium.

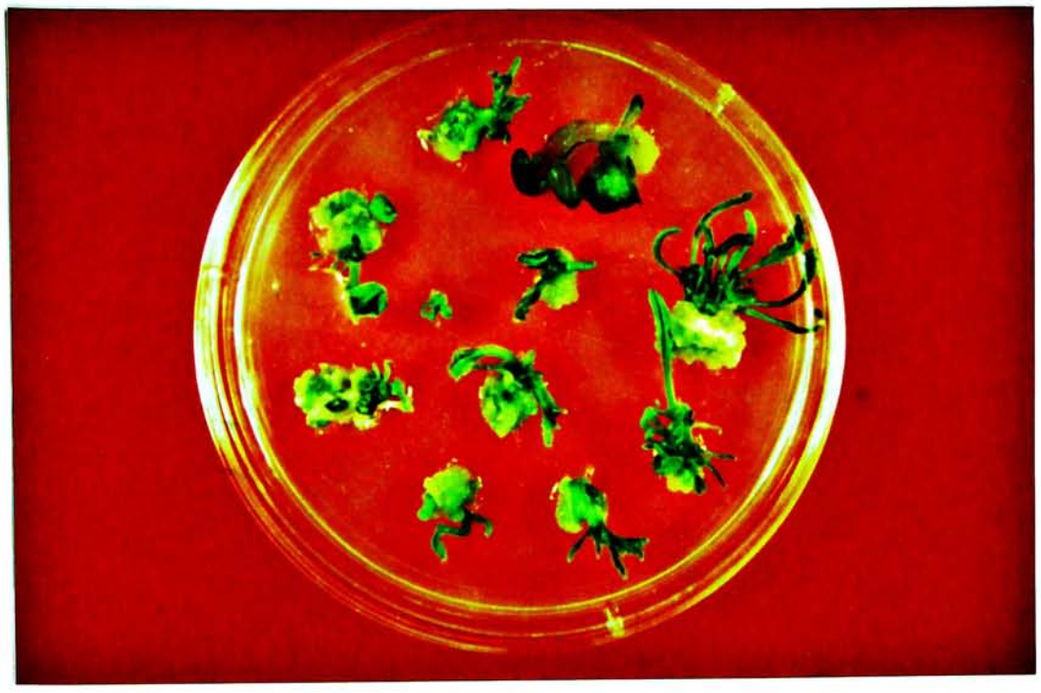
internode stem segment culture, shoot regeneration from the cut ends was observed as shown in Fig. 6.3 and the results are summarized in table 6.2.

Table 6.1 Effects of BA and $AgNO_3$ on shoot regeneration frequency of cotyledon petiole

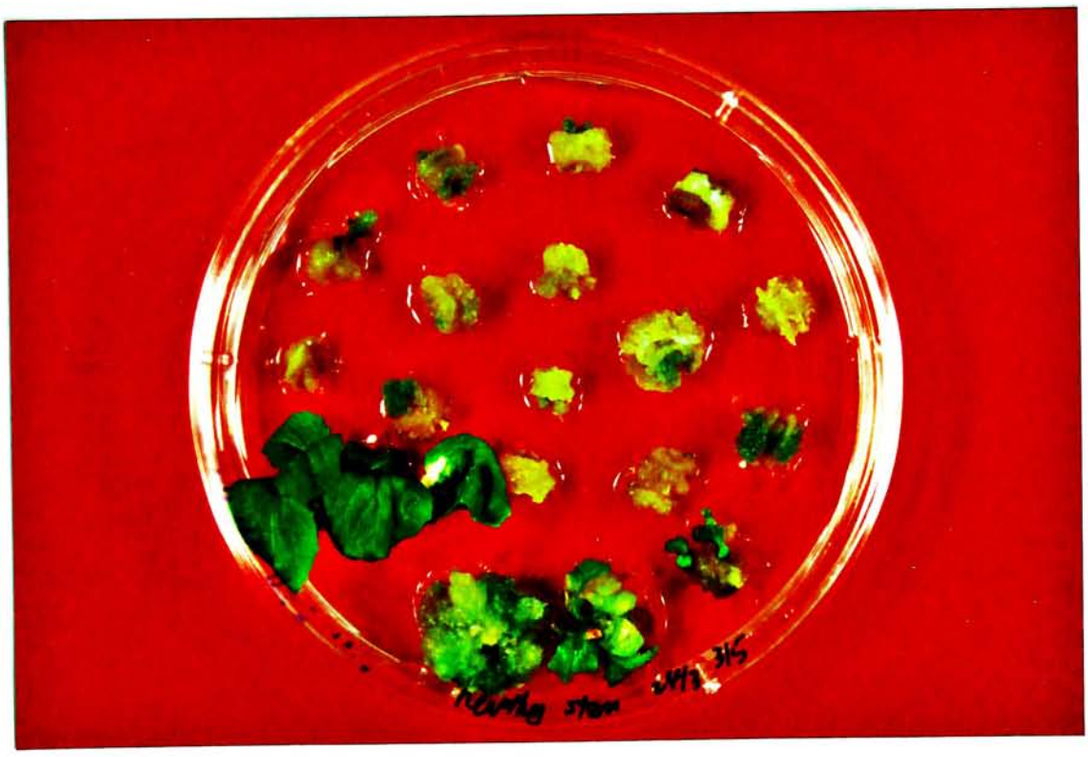
BA (mg/l)	$AgNO_3$ (mg/l)	Number of shoots / Total no. of explant	Regeneration frequency (%)
---	---	---	0
0.5	5	1/40	2.5
1	5	2/40	5
2	5	2/40	5
3	5	---	0
4	5	---	0
5	5	---	0

Table 6.2 Optimal concentration of BA, NAA and $AgNO_3$ on shoot regeneration frequency of internode stem segments

Experiment	BA (mg/l)	NAA (mg/l)	$AgNO_3$ (mg/l)	Number of shoots / Total no. of explants	Regeneration frequency (%)
I	5	0.5	5	18/40	45
II	5	0.5	5	4/30	13



Experiment I



Experiment II

Fig. 6.3 Development of shoots in internode stem segment explants of *B.parachinensis.*

6.3. Discussion

Several reports have showed the successful regeneration of plants from isolated protoplasts as well as from leaf and stem explants of *Brassica napus*, a related species of *B.parachinensis* (Glimelius, 1984; Dunwell, 1981; Pua *et al.*, 1987). Although both protoplasts and explants are suitable materials for gene transfer via co-cultivation with *A.tumefaciens*, the use of explants is preferred because of the faster rate of plant regeneration (Horsch *et al.*, 1985). Also, the protoplast regeneration experiments of *B.chinensis*, a closely related species of *B.parachinensis*, do not produce plants but only calli and roots (Guo and Schieder, 1983).

We conducted experiments with cotyledon petioles, hypocotyl segment and internode stem segment explants of *B.parachinensis* to determine the most suitable type of explants and the optimal concentration of auxin and cytokinin for shoot induction. Since auxin used in the regeneration medium can promote the endogenous production of ethylene in the tissue explants, which is supposed to inhibit shoot promordium formation (Purnhauser *et al.*, 1987), we tried to abolish the ethylene action by using

silver nitrate which is known to be a potent inhibitor of ethylene action in plants (Purnhauser *et al.*, 1987; De Block *et al.*, 1988). We found that internode stem segments generally exhibit a higher frequency of shoot regeneration than hypocotyls and cotyledon petioles (Tables 6.1 and 6.2).

Although a higher frequency of shoot regeneration has been obtained in the internode stem segments, we observed a large variation in the regeneration frequency of 13–45% compared to the steady shoot regeneration frequency of 33.3 ± 1.8% obtained in the experiments using the internode stem segments of *B.napus* (Pua *et al.*, 1987). A reason for this variation may be due to the difference in the growth stage of the shoot culture from which the internode stem segments derived and used in the two experiments. Further experiments should be carried out with internode stem segments of similar growth stage to reduce this error.

Chapter 7
Conclusion

Chapter 7 Conclusion

The present study shows that exogenous application of recombinant TCS protein in 250 μg/ml and 400 μg/ml inhibited the TuMV lesion formation in local lesion host *N.tabacum* by 54% and 82% respectively. A dose response was observed in which less local lesions were found as TCS concentration increased. This dose response were also observed in the systemic host *B.parachinensis* in which the delay in the day of mosaic symptom development increased with the increase in dosage of TCS from 1 to 100 μg/ml. Exogenous application of other RIPs such as PAP, Abrin, Ricin, Modeccin, Gelonin, Momordin, and MAP have also been shown to be effective in inhibiting the infection by various viruses (Tomlinson *et al.*, 1974; Stevens *et al.*, 1981; Kubo *et al.*, 1990).

The mechanism of viral resistance conferred by the exogenous application of TCS or other RIPs may be that during mechanical inoculation of virus, TCS or other RIPs enters the host cells together with the virus and prevents translation of the viral RNA by inhibiting the protein synthesis of the host cells. The possibility that the basic natured

TCS (pI 9.4) binds with the virus and prevents the virus from infecting the cells seems to be low since the exogenous application of cytochrome C (pI 9.6) with similar pI value did not inhibit the local lesion formation by TuMV in *N.tabacum*.

Furthermore, transgenic tobacco plants were produced which expressed TCS mature protein under control of the cauliflower mosaic virus 35S gene promotor, by the *Agrobacterium*-mediated transformation system. Three transgenic plants which were resistance to kanamycin and shown to synthesize TCS protein by Western blotting, are 100% resistant to the mechanical transmission of TuMV. It is considered that endogenous TCS localized in cytoplasm may either bind with the virus after infection or inhibit the viral translation by its ribosome inactivating activity in the host cells.

Transgenic tobacco plants expressing TCS at 0.06–0.1% of total protein tended to have old leaves pale in colour. This may be due to the accumulation of expressed TCS and the potent ribosome inactivating activity of TCS. Nevertheless, since the plants were still capable of

growing, it seems that the endogenous TCS in transgenic tobacco has a differential effect on plant and viral translation. Other study of transgenic tobacco by PAP have the PAP compartmentalized in the cell wall (Lodge *et al.*, 1993). Also, a chimeric gene contained the coding region of Mirabilis jalapa Antimicrobial Protein (Mj-AMP) cDNA extended by 48 nucleotides encoding the vacuolar targetting signal from barley lectin was constructed, and introduced in tobacco recently, which resulted in the subcellular localization of Mj-AMP in transgenic tobacco (De Bolle *et al.*, 1994). These minimize the adverse effect of PAP and Mj-AMP on the host translation and the plants were healthy grown. Hence in the future, it is worthy to transport TCS out of the cytoplasm by similar means or reduce the expression of TCS to its minimal effective level for the increase of the yield of the concerned crop. Since the discovery of board-spectrum virus resistance in transgenic plants expressing PAP has been reported (Lodge *et al.*, 1993), the advantages of using RIP in transgenic plants to inhibit viral infection are the board spectrum of viruses RIP can inhibit and their bio-degradability. Hopefully, with further fine tuning of expression, TCS will become a useful protein to protect various valuable commercial plants from viral infection.

An efficient protocol to regenerate *B.parachinensis* from the internode stem segment explants has also been established in which 13–45% of regeneration frequency was obtained. The next step would be to transform this commercial local species with TCS to find if the transgenic plants become resistant to TuMV infection.

Appendix

A.1. Size of molecular weight markers

A.1.1. λ DNA–*Hin*d III digest marker

The *Hin*d III digest of lambda DNA yields 8 fragments. The cohesive ends of fragments 1 and 4 may be separated by heating to 60°C for 3 minutes.

Fragment	Base pairs
1	23,130
2	9,416
3	6,557
4	4,361
5	2,322
6	2,027
7	564
8	125

A.1.2. λ DNA–*Bst*E II digest marker

The BstE II digest of lambda DNA yields 14 fragments. The cohesive ends of fragments 1 and 4 may be separated by heating to 60°C for 3 minutes.

Fragment	Base pairs
1	8,454
2	7,242
3	6,369
4	5,686
5	4,822
6	4,324
7	3,675
8	2,323
9	1,929
10	1,371
11	1,264
12	702
13	224
14	117

A.1.3. Low molecular weight protein markers

Low molecular weight protein markers consist of six protein markers which may be denatured by heating them to 100°C for 3 minutes.

Protein markers	kiloDalton
Phosphorylase b	94,000
Albumin	67,000
Ovalbumin	43,000
Carbonic anhydrase	30,000
Trypsin inhibitor	20,100
a-lactalbumin	14,400

A.2. References

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